



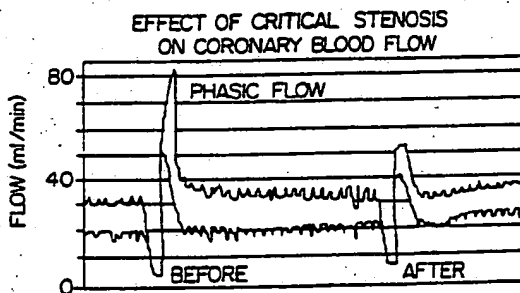
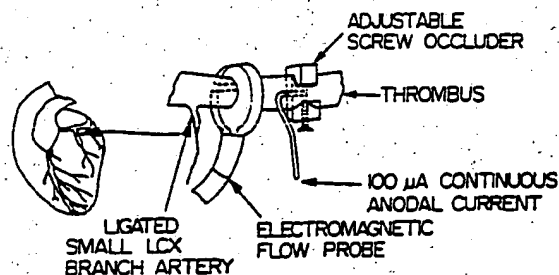
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(54) Title: METHOD AND COMPOSITION FOR TREATING THROMBOSIS

(57) Abstract

The invention provides cyclic peptides which inhibit platelet aggregation without causing prolonged bleeding time. The invention provides RGD or KGD containing peptides which are cyclized and contain hydrophobic moieties adjacent to the carboxy terminus of the RGD sequence. Peptides of this nature are also provided which contain in addition to the hydrophobic moiety an adjacent positively charged moiety. Such peptides have a high affinity for the receptor IIb/IIIa and a low affinity for the fibronectin and vitronectin receptors. Such peptides can be administered in a suitable physiologically acceptable carrier to therapeutically treat thrombosis.



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METHOD AND COMPOSITION FOR TREATING THROMBOSIS

BACKGROUND OF THE INVENTION

5 The invention relates generally to methods of treating thrombosis, and, more particularly, to such methods using peptides.

10 The formation of a blood clot within a blood vessel, a process termed thrombosis, is a serious condition which can cause tissue damage and, if untreated, eventually death. Thrombotic formation is dependent upon platelet aggregation. The interaction of blood platelets with the endothelial surface of injured blood vessels and with other
15 platelets is a major factor in the course of development of thrombi.

20 Various products for dissolving such clots are now available, such as aspirin, dipyridamole and heparin. These products generally kill or remove platelets, which can eliminate the clot, but has the potential serious side effect of causing prolonged bleeding. Moreover, the effect of such products can only be reversed by new platelets being formed or provided.

25 Platelet aggregation is dependent upon the binding of fibrinogen and other serum proteins to the glycoprotein GP IIb/IIIa complex on the platelet plasma membrane. GP IIb/IIIa is a member of a large family of cell adhesion receptors known as integrins, many of which are known to
30 recognize an Arg-Gly-Asp (RGD) tripeptide recognition sequence. Individual receptor specificity is determined by the conformation that the RGD sequence adopts in each individual ligand. Inhibition of GP IIb/IIIa receptor binding, and therefore of platelet aggregation, without
35 inhibition of other cell adhesion receptors would be necessary for the prevention of coronary thrombosis.

There thus exists a need for a composition able to specifically inhibit the platelet aggregation receptor GP IIb/IIIa and to dissolve blood clots without removing or killing platelets and without causing detrimental side effects such as prolonged bleeding. The present invention satisfies this need and provides related advantages as well.

SUMMARY OF THE INVENTION

10 The invention provides cyclic peptides which inhibit platelet aggregation without causing prolonged bleeding time. The invention provides RGD or KGD containing peptides which are cyclized and contain hydrophobic moieties adjacent to the carboxy terminus of the RGD
15 sequence. Peptides of this nature are also provided which contain in addition to the hydrophobic moiety an adjacent positively charged moiety. Such peptides have a high affinity for the receptor IIb/IIIa and a low affinity for the fibronectin and vitronectin receptors. Such peptides
20 can be administered in a suitable physiologically acceptable carrier to therapeutically treat thrombosis.

BRIEF DESCRIPTION OF THE DRAWINGS

25 Figure 1 illustrates the insertion of electromagnetic flow probe, intra-coronary electrode and screw occluder into the left circumflex coronary artery (upper) and shows coronary blood flow before and after adjustment of the critical stenosis (lower).

30 Figure 2 is a dose-response analysis of the relative anti-aggregation potencies of the peptides injected in Example VII.

Figure 3 shows the platelet aggregation values for
35 each peptide dose following time after induction of

coronary thrombosis.

Figure 4 shows the effects of the peptides injected in Example VII on coronary blood flow and thrombosis.

5 Figure 5 shows the effects of the peptides injected in Example VII on hemodynamic responses.

10 Figure 6 shows the platelet and fibrinogen uptake by Gore-Tex grafts in animal 1 injected with the peptide of Example VIII.

Figure 7 shows the platelet uptake rates by Gore-Tex grafts in animal 1 injected with the peptide of Example VIII.

15 Figure 8 shows the hematology parameters in animal 1 for control treatment (shunt 1) and for treatments with the peptide of Example VIII (shunt 2).

20 Figure 9 shows the platelet and fibrinogen uptake by Gore-Tex grafts in animal 2 injected with the peptide of Example VIII.

25 Figure 10 shows the platelet uptake rates by Gore-Tex grafts in animal 2 injected with the peptide of Example VIII.

Figure 11 shows the hematology parameters in animal 2 for control treatment (shunt 1) and for treatments with the peptide of Example VIII (shunt 2).

30 Figure 12 shows the in vivo and ex vivo efficacy of various hydrophobically enhanced peptides in a rabbit model.

DETAILED DESCRIPTION OF THE INVENTION

The invention provides cyclic peptides which inhibit platelet aggregation without causing prolonged bleeding time. In one embodiment, peptides are also provided which are cyclic and contain the sequence $X_a GDX_b^*$ wherein X_a is a positively charged amino acid, X_b is a hydrophobic moiety and $*$ is a positively charged moiety. These peptides are effective in inhibiting platelet aggregation and can therefore advantageously be used to dissolve blood clots as well as prevent inappropriate growth of vascular smooth muscle cells and arterial graft occlusion. Unexpectedly, such treatment does not cause the concomitant significant prolonged bleeding which has limited the usefulness of other anti-thrombotic agents. The use of such peptides is therefore a significant improvement over conventional therapy, including such therapy utilizing other RGD-containing peptides.

As used herein, references to "Arg-Gly-Asp containing peptides" or "RGD peptides" are intended to refer to peptides having one or more Arg-Gly-Asp containing sequences which may function as binding sites for a receptor of the "Arg-Gly-Asp family of receptors", i.e., those recognizing and binding to the Arg-Gly-Asp sequence. It is understood that functional equivalents of Arg-Gly-Asp such as Lys-Gly-Asp (KGD) or chemical structures other than amino acids which functionally mimic the Arg-Gly-Asp tripeptide sequence are also included within this definition. While the Arg-Gly-Asp sequence and its functional equivalents have been found to necessarily be invariant in order to retain the binding activity, the composition of the remaining peptide as well as any other chemical moiety present in conjunction with the peptide may vary without necessarily affecting the activity of the binding site. Where specific chemical structures or sequences beyond the Arg-Gly-Asp sequence are presented, it is intended that

various modifications which do not destroy the function of the binding site are to be encompassed without departing from the definition of the peptide.

5 As used herein, the term "bridge" refers to a chemical bond between two amino acids, amino acid derivatives or other chemical moieties in a peptide other than the amide bond by which the backbone of the peptide is formed unless the amide bond cyclizes the peptide to form a lactam.

10 As used herein, the term "peptide bond" or "peptide linkage" refers to an amide linkage between a carboxyl group of one amino acid and the α -amino group of another amino acid.

15 As used herein, the term "peptide" is intended to include molecules containing amino acids linearly coupled through peptide bonds. Such peptides may additionally contain amino acid derivatives or non-amino acid moieties. The amino acids can be in the L or D form so long as the
20 binding function of the peptide is maintained. Such peptides can be of variable length, preferably between about 4 and 200 amino acids, more preferably between about 7 and 35 amino acids most preferably, about 19 amino acids. The term amino acid refers both to the naturally occurring
25 amino acids and their derivatives, such as TyrMe and PheCl, as well as other moieties characterized by the presence of both an available carboxyl group and amine group. Non-amino acid moieties which can be contained in such peptides include, for example, nitrogen containing moieties such as
30 amines, acyl groups or amino acid mimicking structures. Mimicking structures are those structures which exhibit substantially the same spatial arrangement of functional groups as amino acids but do not have both the α -amino and α -carboxyl groups characteristic of amino acids.

35 As used herein, the term "cyclic peptide" refers to a

peptide having an intramolecular bond between two non-adjacent amino acids within a peptide. The intramolecular bond includes, but is not limited to backbone to backbone, side-chain to backbone and side-chain to side-chain cyclizations. Various amino acid derivatives and chemical moieties can participate in such bonds, including, for example, Pen, Pmp and Pmp analogues and Pmc and Pmc analogues. Pmc is also known as amino-Pmp (am-pmp).

As used herein, the terms "not prolonging bleeding time" or "not substantially prolonging bleeding time" refers to a bleeding time which is substantially the same as that obtained from an untreated animal. Thus peptides which do not prolong bleeding time are those which when administered to an animal do not extend the bleeding time by more than a factor of two as measured by assays such as that provided in Example VII 6., infra.

The one-letter and three-letter abbreviations for amino acids and other moieties used herein are given as follows:

	A	Ala	Alanine
		α -ABA	α -Amino isobutyric acid
25	R	Arg	Arginine
	N	Asn	Asparagine
	D	Asp	Aspartic acid
		Cha	Cyclohexyl-alanine
		Cit	Citrulline
30	C	Cys	Cysteine
	Q	Gln	Glutamine
	E	Glu	Glutamic acid
	G	Gly	Glycine
	H	His	Histidine
35		Hpa	Homophenylalanine
	I	Ile	Isoleucine
	L	Leu	Leucine

	K	Lys	Lysine
	M	Met	Methionine
		napA	naphthyl-alanine analogues
5		O-n-butyl-Tyr	O-n-butyl-tyrosine
		O-n-hexyl-Tyr	O-n-hexyl-tyrosine
		Orn	Ornithine
		P-amino-Phe	para-amino-phenylalanine
		Pas	6,6-Cyclopentamethylene-2-Aminosuberic acid analogues
10		Pen	Penicillamine
	F	Phe	Phenylalanine
		PheCl	para-chloro-phenylalanine
		Phg	phenylglycine
		P-iodo-Phe	para-iodo-phenylalanine
15		Pmc	amino- β , β -pentamethylene- β -mercaptopropionic acid
		Pmp	β , β -pentamethylene- β -mercaptopropionic acid analogues
		P-nitro-Phe	para-nitro-phenylalanine
20	P	Pro	Proline
	S	Ser	Serine
		SuccAla	Succinyl-alanine
	T	Thr	Theonine
	W	Trp	Tryptophan
25	Y	Tyr	Tyrosine
		TyrMe	O-methyl-Tyrosine
	V	Val	Valine
		2-Nal	(2-naphthyl) alanine
		3,5-diiodo-Tyr	3,5-diiodo-tyrosine

30 As used herein, the term "hydrophobic" is intended to include those amino acids, amino acid derivatives, amino acid mimics and chemical moieties which are non-polar. Hydrophobic amino acids include Phe, Val, Trp, Ile and Leu. Other hydrophobic moieties useful in the invention are

35 TyrMe, PheCl and ChA O-n-hexyl-Tyr, 3,5-diiodo-Tyr, Hpa, 2-Nal, O-n-butyl-Tyr, p-nitro-Phe, Phg, P-iodo-Phe, P-amino-

Phe and Cit.

As used herein, the term "positively charged moiety" refers to those amino acids, amino acid derivatives, amino acid mimics and chemical moieties which are positively charged. Positively charged amino acids include, for example, Lys, Arg and His and Orn.

Although the invention will be described with reference to RGD receptor binding peptides, it is understood that functional equivalents known to those skilled in the art can be substituted for the RGD sequence without departing from the spirit of the invention. One skilled in the art will be able to use such functional equivalents to practice the invention described herein.

It is now well-established that the amino acid sequence RGD is the cell binding site in a number of proteins, including for example, fibronectin, vitronectin and type IV collagen. The RGD binding site is recognized by a family of cell surface receptors, termed integrins. Platelets contain a large repertoire of RGD-cell surface receptors, each of which recognizes one or more RGD containing ligands to perform various physiological functions. GP IIb/IIIa is one such integrin receptor found in platelets. The ligands recognized by this receptor include fibrinogen and other serum proteins. GP IIb/IIIa is primarily responsible, through interaction with other platelets to form aggregates and through interactions with the endothelial surface of injured blood vessels, for the development of coronary artery thrombosis. When provided in soluble form, RGD peptides can inhibit cell attachment or platelet aggregation through competition with other RGD containing ligands. See for example US Pat NOS. 4,578,079, 4,517,686, 4,792,525 and 4,683,291, which are incorporated herein by reference.

Because prolongation of bleeding time can be an undesirable side effect of thrombolytic therapy the present peptides which do not have this side effect are extremely useful. The effect of a peptide on bleeding time can be easily determined by one skilled in the art using for example, a protocol as described in Example VII 6. Hemodynamic Responses.

Unexpectedly, the presence of a positively-charged moiety in the "+4 position" of the RGD binding site, i.e., the position adjacent to the residue in the X position of the sequence RGD_X, confers the characteristic of not prolonging bleeding time to platelet aggregation inhibiting peptides. Illustrative of such a peptide is Ac-CNPRGD(Y-OMe)RCNH₂ ("8X"). Such a positive charge can, for example, result from the presence of a positively charged amino acid, such as Arg or Lys homo-Arg or ornithine, in the +4 position. Alternatively, an amino acid derivative or amino acid mimic having a positive charge in the +4 position can produce the desired effect. In addition, a positively charged chemical moiety which is spatially arrayed so as to occupy substantially such a position can also confer the characteristic. Such a moiety need not be linearly arrayed in the +4 position so long as its positive charge occupies substantially the same spatial site as that occupied by the guanidino side chain 8X, supra.

The peptides of the present invention can be synthesized by any of the suitable methods well known in the art including methods of chemical synthesis. Preferably, the linear sequence is synthesized using commercially available automated peptide synthesizers such as those manufactured by Applied Biosystems, Inc., Foster City, CA. The material so synthesized can be precipitated and further purified, for example by high performance liquid chromatography (HPLC). Although a purity of greater

than 95 percent for the synthesized peptide is preferred, lower purity may be acceptable.

To obtain one of the enhanced peptides of the present invention which have a high potency for inhibiting platelet aggregation and which do not cause prolonged bleeding when administered to an animal, the synthesized peptide is cyclized using methods well known in the art. Cyclization can be achieved where the peptides contain two sulphur-containing amino acids, or other moieties through a disulfide bond. Examples of useful sulphur-containing moieties are Cys and Pen and Pmp. Alternatively, cyclization can be accomplished through the formation of a peptide bond or alkyl bridge structures using, for example, Pas. Where the residues contain sulfhydryls, a disulfide bridge can be formed by oxidizing a dilute aqueous solution of the peptides with $K_3[F_6(CN)_6]$. Other residues, such as the chemical moieties Pmp and Pas, can create a bridging structure through a disulfide bond between Pmp and Cys (or similar structures) and an alkyl bond between Pas and an amino acid methylene moiety (or similar structures). Other means of cyclizing, which are known in the art, can also be utilized.

The cyclized peptides of the present invention can also be prepared by forming a peptide bond between non-adjacent amino acid residues. A procedure for forming such peptide bond is provided in Schiller et al., Int. J. Peptide Protein Res. 25:171 (1985), which is incorporated herein by reference. Briefly, cyclic peptides can be synthesized on the Merrifield resin by assembling the peptide chain using N^{α} -Fmoc-amino acids and Boc and tertiary-butyl protein.

Side-chain to side-chain cyclizations can be performed by the above procedure or using N^{α} -Boc-amino acids together with OFm/Fmoc side-chain protection for Asp and Lys

residues as described by Felix et al, Int. J. Peptide
Protein Res. 31:231 (1988), which is incorporated herein by
reference. Alternatively, side-chain to backbone
cyclizations can be performed using this procedure or in
5 combination with the procedure described in the preceding
paragraph.

Alternative methods of making cyclized peptides are
disclosed, for example, in WO 91/01331 entitled "SMALL
10 CYCLIC PEPTIDE AGGREGATION INHIBITORS," published 7
February 1991. As described therein, for example,
Bromoacetyl-Gly-Arg(g-2,2,5,7,8-pentamethylchroman-8-
sulfonyl)-Gly-Asp(beta-t-butyl)-Cys(S-triphenylmethyl)-O-
(polymer resin) can be prepared using standard solid phase
15 peptide synthesis utilizing fluorenylmethoxycarbonyl (Fmoc)
protecting group chemistry on a p-alkoxybenzyl alcohol
resin. Repeated treatment of the resin bound peptide with
a 1% solution of trifluoroacetic acid in dichloromethane
results in cleavage of the S-triphenylmethyl group as
20 evidenced by the bright yellow of the solution. Treatment
is continued until dissipation of the yellow color (ca. 1.5
L of the cleavage solution is required per gram of resin
bound peptide). After complete cleavage of the S-
triphenylmethyl group, the resin bound peptide is washed
25 several times with a 5% solution of N-methylmorpholine in
N,N-dimethylacetamide and then shaken in pure N,N-
dimethylacetamide for 12 hours to complete the cyclization.
Treatment of the cyclized resin bound peptide with
trifluoroacetic acid containing (v/v) 1% phenol, 1% anisole
30 and 1% ethanedithiol effects concomitant cleavage of the
remaining protective groups and cleavage of the desired
product from the resin, which can then be purified, for
example by HPLC using a 4.6 mm x 250 mm column containing
10 micron, 300 Angstrom pore size C-18 packing. The
35 elution of the column was with an acetonitrile 0.1% aqueous
trifluoroacetic acid gradient going from 0% - 40%
acetonitrile linearly over 80 minutes.

Peptides included in the present invention can contain a hydrophobic moiety adjacent the carboxy terminus of the RGD sequence. The hydrophobic moieties can have a range of structural types and hydrophobicities. Appropriate hydrophobic moieties include for example Phe, Trp, Val, Ile, Leu, PheCl, TyrMe, and ChA O-n-hexyl-Tyr, 3,5-diiodo-Tyr, Hpa, 2-Nal, O-n-butyl-Tyr and the like. Such peptides can be represented as $X_1X_2X_3X_4GDX_5X_6X_7$ wherein X_5 is a hydrophobic moiety; X_2 and X_6 are moieties capable of forming a bridge, such as a disulfide bridge, alkyl bridge or a peptide bond. Representative of these moieties are Cys, Pen, Pmp, Pmc and Pas. X_1 and X_7 are 0 to 20 amino acids. When the number of residues of X_1 is one, X_1 is preferably a Gly; when the number of residues is greater than one, the carboxy terminal residue is preferably a Gly. X_3 is 1 to 10 amino acids, preferably with His or Pro as the carboxy terminal residue, a positively charged amino acid, such as Arg or Lys. Specific peptides of this nature include RPenGRGDWPCR, GPenGHRGDLRCA, RPenGHRGDWRCR, RPenGHRGD(ChA)RCR, PmpGHRGDLRCA, G(dPen)GHRGDLRCA, R(am-Pmp)GHRGDWRCR, R(am-Pmp)GHRGD(TyrMe)RCR, R(am-Pmp)GHRGD(PheCl)RCR, R(am-Pmp)GHRGDLRCR, R(am-Pmp)GHRGDLRCR, R(t-but-am-Pmp)GHRGDLRCR, Ac-CNPRGD(Y-OMe)RCNH₂, Ac-CNPKGD(Y-OMe)RC-NH₂, Ac-CNPRGD(O-N-Butyl-YORC-NH₂.

Alternatively, the peptides can be represented as a composition of matter comprising an $-X_aGDX_b^*-$ binding site containing cyclic peptide wherein X_a is a positively charged amino acid, X_b is one or more amino acid or other moiety so as to confer platelet aggregation inhibiting activity and wherein * is a positively charged moiety located in appropriate spatial proximity to said binding site so that upon administration of the composition to an animal, bleeding time is not substantially increased over that of an untreated animal.

Peptides having sequences other than those specifically identified herein are also included in the invention provided they exhibit the requisite functional criteria. Peptides which have high anti-thrombotic activity and which do not prolong bleeding time can be synthesized and tested using the teachings described herein. Such peptides once synthesized can be tested for anti-thrombotic activity using, for example, the platelet aggregation assays described in Example V. The relative affinity of the peptide for the various integrin receptors can be tested using, for example, the liposome binding assay of Example IV or the ELISA assays of Example III. Peptides exhibiting high potency for inhibiting platelet aggregation and also showing high affinity for GPII_bIII_a and low affinity for the fibronectin and vitronectin receptors should be selected to determine their effect on bleeding time. Animal models such as the baboon models or rabbit models described in Examples VIII and IX can be used to assess bleeding time. After the peptides are administered, the effect can be monitored by cutting the animal and allowing it to bleed onto an absorbent towel or tissue. The length of time until the bleeding stops should be compared to the animals bleeding time without peptide administration. Peptides which fall within the teachings described herein will not increase the bleeding greater than about 2-fold over that of an untreated control. Thus, one skilled in the art can use the teachings of the invention to make and test a variety of peptides having the generic structures described herein.

In another aspect of the invention, peptides are provided that possess relatively high affinity for the receptor IIb/IIIa and low affinity for the fibronectin and vitronectin receptors. Such IIb/IIIa affinity can be determined, as for example, by a liposome attachment assay, as described in Examples IV and VI or in a platelet aggregation assay as described in Example V. Peptides

characterized by high affinity for IIb/IIIa will have an IC_{50} as measured under the assay conditions provided in Examples IV and VI of less than about $10\mu\text{m}$, preferably less than about $1\mu\text{m}$, more preferably about $0.1\mu\text{m}$.

5 Alternatively, affinity for IIb/IIIa as characterized in Example V will have an IC_{50} of less than about $10\mu\text{m}$, preferably less than about $1\mu\text{m}$, more preferably about $0.1\mu\text{m}$. Fibronectin receptor affinity and vitronectin receptor affinity can be determined as, for example, by the methods

10 detailed in Examples III and VI, and IV and VI respectively. Using these assays, under the conditions described, peptides having low affinity for the fibronectin receptor will have an IC_{50} of greater than about $0.1\mu\text{m}$, preferably greater than about $1\mu\text{m}$, more preferably

15 greater than about $10\mu\text{m}$; low affinity for the vitronectin receptor is greater than about $1\mu\text{m}$, preferably greater than about $10\mu\text{m}$, more preferably greater than about $100\mu\text{m}$. It is thus possible to screen various peptides in order to determine their inhibitory concentrations, and

20 therefore binding affinities, and to select those having high affinity for the IIb/IIIa and low affinity for the fibronectin and vitronectin receptors.

The invention also provides peptides which

25 differentially inhibit the binding of various ligands to GPII_b/III_a, and do not result in a prolongation of bleeding time. For example, the peptide designated herein as 8X inhibits the binding of fibrinogen to GPIIb/IIIa more than it inhibits the binding of van Willebrand factor to the

30 same integrin.

The peptides of the present invention can be utilized to effectively eliminate thrombotic conditions by administering to a mammal exhibiting thrombosis a

35 therapeutically effective amount of the peptide in a suitable physiologically acceptable carrier. Effective amounts will be 1 to 50 mg/kg/hr body weight, preferably

about 1 to 5 mg/kg body weight. Appropriate effective amounts can be easily determined by those skilled in the art. The peptide can be administered in a variety of ways, as for example, by infusion or injection. Length of treatment can be determined by monitoring effect.

The claimed subject matter includes a composition of matter comprising a cyclic RGD containing peptide having a hydrophobic moiety adjacent the carboxy terminus of the RGD sequence. In one embodiment, a composition of matter comprising a cyclic peptide having the sequence $X_1X_2X_3X_4GDX_5X_6X_7$, wherein X_2 and X_6 comprise moieties capable of forming a bridge, X_3 is one or more amino acids and X_1 and X_7 are zero to 20 amino acids and X_5 comprises a hydrophobic moiety and X_4 is a positively charged amino acid. In another embodiment, a composition of matter comprising an RGD-binding site containing cyclic peptide having platelet aggregation inhibiting activity without substantially prolonging bleeding time. In a further embodiment, a composition of matter comprising an $-X_aGDX_b^*$ binding site containing cyclic peptide wherein X_a is a positively charged amino acid, X_b is one or more amino acid or other moiety so as to confer platelet aggregation inhibiting activity and wherein $*$ is a positively charged moiety located in appropriate spatial proximity to said binding site so that upon administration of the composition to an animal, bleeding time is not substantially increased over that of an untreated animal. Other peptides include a peptide selected from the group consisting of:

30

RPenGRGDWPCRGPenGHRGDLRCARPenGHRGDWRCRRPenGHRGD(Cha)RCRPmpGHRGDLRCA

35

G(dPen)GHRGDLRCAR(am-Pmp)GHRGDWRCRR(am-Pmp)GHRGD(TyrMe)RCR

R(am-Pmp)GHRGD(PheCl)RCR

R(am-Pmp)GHRGDLRCR

R(am-Pmp)GHRGDLRCR

R(t-but-am-Pmp)GHRGDLRCR

Ac-CNPRGD(Y-OMe)RCNH₂

Ac-CNPKGD(Y-OMe)RC-NH₂

Ac-CNPRGD(O-N-Butyl-YORC-NH₂

These compositions can be combined with a physiologically acceptable carrier and used to treat thrombosis and vascular graft occlusion.

The following Examples are intended to illustrate but not limit the invention.

EXAMPLE I

Peptide Synthesis

Peptides were synthesized on an automated peptide synthesizer (Model 430A, Applied Biosystems, Foster City, California USA) using optimized n-methyl pyrrolidone chemistry on PAM resin as recommended by the manufacturer. Cleavage of the peptides from the resin was achieved with 100% hydrogen fluoride. The peptides were further purified by HPLC using a VYDAC reverse phase C₁₈ column with 0 to 60% acetonitrile gradients. Peptides were used only if found to be $\geq 98\%$ pure.

For Pen cyclization, 611 mg of the peptide synthesized as described above were dissolved in 4 liters of water that had been previously boiled and allowed to cool. Immediately prior to addition of the peptide, nitrogen was bubbled through the water for 45 minutes. After the peptide was dissolved, a solution of 0.1 $\mu\text{g/ml}$ of potassium ferrous cyanide K₃[Fe(CN)₆] in water was added dropwise to the stirred peptide solution until the yellow color

persisted for 5 minutes (approximately 5 ml). The pH of the solution was held at 7.0 throughout this procedure by addition of NH_4OH . The solution was allowed to stand for 20 hours under low vacuum and then lyophilized. Excess $\text{K}_3[\text{Fe}(\text{CN})_6]$ was removed by passing the cyclized material over a Sephadex G-15 column (1.8 x 120 cm). The peptide was purified by reverse phase HPLC using a Waters BondapakTM C_{18} column (3 x 30 cm; 10 μm packing) (Waters Assoc., Milford, MA). The peptide was loaded on the column in buffer A (20 mM ammonium acetate at pH 7.5) and eluted with a gradient of buffer B consisting of 60% acetonitrile and 40% buffer A. Eluted fractions were tested for their ability to inhibit receptor binding.

The major peak obtained from the C_{18} column constituted 90% of recovered peptide and was deduced to be a monomeric cyclic peptide because it was retained on the column for the length of time predicted for that sequence and because the uncyclized material and multimeric forms were well separated from the main peak.

EXAMPLE II

Receptor and Ligand Purifications

Receptors were purified according to the procedures of Pytela et al. (Methods Enzymol. 144:475 (1987)), incorporated herein by reference. Briefly, vitronectin receptor (Vn-R) was purified by RGD peptide-affinity chromatography from (100 mM) octyl glucoside (OG) extracted human placenta. After extraction, the suspension was filtered over a Sepharose 6B column and then applied to a GRGDSPK column. Except where stated, all procedures were carried out at 4°C. The peptide column was washed with three volumes of Tris-buffered saline (TBS) containing 1 mM Ca^{2+} and 25 mM OG and then with TBS containing 1 mM Ca^{2+} and 25 mM octyl thioglucoside (OTG) at room temperature.

Elution of bound receptor was achieved at room temperature with TBS containing 20 mM EDTA and 25 mM OTG. Finally, Ca^{2+} and Mg^{+2} were added to eluted fractions to achieve final concentrations of 1 mM for both ions.

5 Fibronectin receptor (Fn-R) was similarly purified from (100 mM) octyl glucoside-extracted human placenta using a procedure identical to that for the Vn-R up to and including the initial Sepharose chromatography step. The Sepharose 6B column flow-through was brought to 2 mM Mn^{+2}
10 and the resulting solution was run over a 110 kd fibronectin fragment-affinity column. Washing and elution steps were identical to those used in purifying vitronectin receptor.

15 Platelet glycoprotein IIb/IIIa was purified from outdated human platelets. Briefly, the platelets were washed 3 times with 10 mM tris-HCl, 150 mM NaCl (TBS), 1 mM EDTA, pH 7.5, and centrifuged at 2000 x g to pellet cells. Cells are lysed in 5 pellet volumes of TBS, 1% Triton X-
20 100, 1 mM Ca_2Cl_2 , and followed by centrifugation at 30,000 x g. The supernatant fraction is collected and the supernatant is loaded onto a concanavalin-A column, previously equilibrated in TBS, 1 mM Ca_2Cl_2 , 0.1% Triton, 0.05% NaN_3 and eluted with 0.2 M α -methylmannoside.
25 Fractions are pooled and loaded onto a heparin-agarose column. The flowthrough is collected and concentrated on an Amicon YM 30 filter to a volume of approximately 5-10 ml. The concentrate is then applied to an S-300 column (500 ml) and 6 ml fractions are collected. The GPII_bIII_a
30 containing fractions are collected, pooled and stored at -80°C.

The purification of fibrinogen is conducted essentially as described by Lipinska et al., (J. Lab. Clin.
35 Med. 507 (1974)). Briefly, a 0.3% w/v/ solution of human fibrinogen (Kabi #5302) is dissolved in 150 mM NaCl.

Saturated $(\text{NH}_4)_2\text{SO}_4$ is added dropwise with stirring to the fibrinogen solution to obtain about 16% saturation. The precipitate is spun down in appropriate size bottles at 2000 x g. The supernatant is decanted and the precipitate
5 resuspended in 150 mM NaCl (approximately 50% of the original volume). NH_4SO_4 is again added dropwise to obtain 16% saturation. The suspension is spun down and the precipitate is resuspended in Tris-saline in a minimal volume (approximately 5% of the original volume). Any
10 remaining insoluble material is spun down at 2000 rpm in a Sorval type centrifuge and the fibrinogen supernatant is decanted and dialyzed overnight at 4°C against Tris-saline. Characterization of the fibrinogen is by the Bradford protein assay, SDS-PAGE, and/or Western blotting using well
15 known standard procedures.

EXAMPLE III

Enzyme-Linked Immunosorbent Assays (ELISA)

20 1. Human Vitronectin-Vitronectin Receptor ($\alpha_v\beta_3$) ELISA Assay

Human vitronectin (Vn) is isolated from human plasma and purified by affinity chromatography by the method of
25 Yatohgo et al., (Cell Structure and Function 13:281-292 (1988)).

The purity of each receptor was assessed with SDS-PAGE under reducing and non-reducing conditions. Each receptor
30 was flash-frozen in liquid nitrogen and stored frozen until use.

2. Fibronectin Receptor (Fn-R) ELISA Assay

35 Peptide binding to purified Fn-R was determined by using a competitive enzyme-linked immunosorbent assay

(ELISA) in which fibronectin is immobilized and the binding of solubilized Fn-R and the binding of solubilized FN-R, in the presence of various concentrations of peptide analogue, is detected with a polyclonal anti-Fn-R antibody followed by a labelled anti-rabbit IgG conjugate.

Microtiter plates were coated with 110 μ l of human fibronectin (at 2 μ g/ml) in TBS. The plates were washed three times with TBS that contained 0.05% Tween 20. 50 microliters of receptor in TBS containing 20 mM octylglucoside and 2 mM NmCl_2 was added to each well. 50 microliters of peptide in the same buffer was then added in 10-fold serial dilutions. The plates were incubated for three hours at room temperature, washed with 200 μ l of the above TBS-Tween buffer. 100 μ l of affinity-purified rabbit anti-human fibronectin receptor antibody was added to the wells and the plates were incubated for an additional two hours, washed twice with TBS-Tween and then distilled water. Affinity-purified goat anti-rabbit IgG conjugated to horseradish peroxidase (100 μ l) was then added to each well. Bonding reactions were incubated for 16 hours at room temperature. The following day, the plates were washed twice with TBS-Tween and then distilled water. 100 μ l of substrate mixture (10 mg O-phenylenediamine in 25 ml 0.1 M citrate-phosphate buffer, pH 5.0, plus six microliters of 30% H_2O_2) was added to the plates and allowed to develop. The development process was stopped by adding 50 μ l of 4N H_2SO_4 to each well.

3. Fibrinogen - GPIIb/IIIa Receptor ELISA (Fg/IIb/IIIa)

Microtiter plates, 96 wells were coated (Type Nunc 1 MaxisporTM) with 10 μ g/ml purified fibrinogen (100 μ l/well), and allowed to stand overnight at 4°C. The plates were washed three times with PBS Tween, 0.137 M NaCl, 0.003 M KCl, 0.008 M Na_2HPO_4 , pH 7.4 at room temperature, 0.05%

Tween-20 and blocked for 1 to 2 hours at room temperature with 200 μ l/well TNCNT (which is 0.5% BSA, 20mM Tris, pH 7.5 at room temperature, 120mM NaCl, 0.2% NaN_3 , 2mM CaCl_2 , 0.05% Tween 20, [Calbiochem RIA grade or better]) on a plate shaker. The plates were again washed three times with PBS/Tween and 50 μ l of sample in TNCNT is added. the mixture was incubated for 15 minutes at room temperature on a plate shaker. The stock solution of purified GPII_bIII_a receptor from human platelets, (0.4 - 1.0 mg/ml GPII_bIII_a in 0.1% Triton X-100, 1mM CaCl_2 , 20 mM Tris, 150 mM NaCl, 0.05% NaN_3 in 0.3 M N-acetyl glucosamine pH 7.5, stored at -70°C), was reconstituted in TNCNT. Fifty μ l of this diluted GPII_bIII_a was then added to each well and incubated on a plate shaker at room temperature. After one hour, the plates were washed four times with PBS/Tween and 100 μ l of a polyclonal or monoclonal antibody specific for GPIII_a such as AP3 (1 μ g/ml) (See e.g. Newman et al., Blood, 65:227-232 (1985)) and ELISA buffer (PBS, 0.5 BSA, 0.05% Tween 20, 0.01% Thimerasol) was added. After one hour incubation at room temperature on a plate shaker, the samples were washed 4 times with PBS/Tween. 100 μ l of GAMHRP (horse radish peroxidase conjugate of goat anti-mouse IgG (Pel-Freeze Cat. 715305-1) dissolved in ELISA buffer) previously diluted to 1:10,000 was then added and incubated 1 hour at room temperature on a plate shaker. Samples were then washed 4 times with PBS/Tween and 100 ml OPD/ H_2O_2 substrate was added (OPD/ H_2O_2 substrate: dissolve 10 mg o-phenylenediamine in 15 ml phosphate/citrate buffer, kept at room temperature, in a 50 ml FalconTM tube covered with foil; just before use, 6.25 μ l of 30% H_2O_2 is added to give a final solution of 0.67 mg OPD/ml in 0.0125% H_2O_2). (The phosphate/citrate buffer consists of 16 mM Citric Acid, 50 mM Na_2HPO_4 , pH 5.0). The color developed within 3 to 20 minutes and the reaction was stopped with 100 μ l 1 M H_2SO_4 . The optical density at 492 nm vs 405 nm was recorded and IC_{50} values are determined.

4. Vitronectin-Vitronectin Receptor ELISA (Vn/VnR)

- Anti-GPII_bIII_a monoclonal antibodies specific for human GPIII_a was prepared by the method of Newman et al. (Blood, 65:227-232 (1985)), or a similar procedure. This mouse Mab is specific for the β_3 subunit of the vitronectin receptor. Rabbit Fab 2 anti-mouse Fc fragment horse radish peroxidase conjugate (anti-MuFc HRP) was obtained from PelFreeze (Cat. No. 715305-1).
- 10 Maxisorp microtiter plates were coated with 2 $\mu\text{g/ml}$ human vitronectin dissolved in PBS (50 ml/well) and stored overnight at 4°C. The plates were washed two times with PBS-0.05% Tween-20 (wash buffer) and blocked by incubating with about 150 $\mu\text{l/well}$ of assay buffer (1% BSA (RIA grade or better) in 50 mM Tris-HCl, 100 mM NaCl, 1 mM MgCl_2 , 15 CaCl_2 , MnCl_2 pH 7.4) for 60 minutes. Dilutions of standards were prepared and putative inhibitors (Table 2) were dissolved in assay buffer. The blocked plates were emptied and 20 $\mu\text{l/well}$ of inhibitor or standard solution was added to each well. Twenty-five μl of a 30 $\mu\text{g/ml}$ solution of purified $\alpha_v\beta_3$ in assay buffer was pipetted into the coated plate. The final concentration of receptor in the assay well was about 15 $\mu\text{g/ml}$. The plate was incubated on a shaker for 60 minutes. Meanwhile, for each microtite 25 plate, 6 ml buffer solution containing 1.5 $\mu\text{g/ml}$ of mouse monoclonal antibody specific for β_3 is prepared. To this solution was added the secondary antibody, which is anti-mouse-Fc-HRP antibody conjugate. For example, for one plate, 6 ml of a 1.5 $\mu\text{g/ml}$ mouse Mab solution was prepared to which was added 1 μl of anti-mouse-Fc-HRP antibody 30 stock, (this represents a 1:6000 dilution of the antibody - HRP conjugate). This mixture was allowed to incubate during the receptor-inhibitor incubation. The assay plates were washed 4 times with PBS-Tween and 50 $\mu\text{l/well}$ of the 35 antibody mixture was then pipetted into the plate for a 60 minute incubation. The plate was washed 4 times and the

color reaction was developed with 50 μ l/well of 0.67 mg/ml o-phenyldiamine in PBS containing 0.012% H_2O_2 . Alternatively, 16 mM citric acid, 50 mM Na_2PO_4 at pH 5.0 can be used as a substrate buffer. The reaction is stopped with 50 μ l/well 1 M H_2SO_4 . The plates were read at 492-405 nm and the data analyzed by four-parameters fit.

5. von Willebrand Factor - GPII_bIII_a Receptor ELISA
(vWf/II_bIII_a)

Microtiter plates were coated with 1.0 μ g/ml GPII_bIII_a, prepared by the method of Fitzgerald et al., (Anal. Biochem. 151:169-177 (1985)) and allowed to incubate overnight in coat buffer. The plates were then washed three times in wash buffer (0.05% Tween 20 in PBS) and 150 μ l of assay buffer was added and allowed to incubate for 1-2 hours at room temperature on plate shaker. The plates were washed three times and 50 μ l of 2x inhibitor in assay buffer (Assay buffer: 0.5% BSA/50 mM Tris, 100 mM NaCl, 1.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, 1.0 mM $MnCl_2$; coat buffer was the same but without BSA) was added. Fifty μ l of 4.0 μ g/ml vWF (prepared as described by Ledford et al., Thrombosis and Hemostasis, 64(4):569-575 (1990)) in assay buffer was then added and allowed to incubate for one hour at room temperature on plate-shaker. The plates were washed three times and the antibody mixture was added (1:5000 of mouse anti-vWF and 1:5000 of rabbit-anti-mouse-Fc-HRP, both commercially available) in assay buffer and incubated for 1 hour at room temperature on plate-shaker. Plates were again washed three times and 100 μ l of substrate solution (10 mg OPD, 6.5 μ l H_2O_2 , 15 ml phosphate citrate buffer) was added and incubated at room temperature. The color change of OPD/ H_2O_2 reagent was read at 492 nm with a 405 nm reference wavelength on the filter photometer.

EXAMPLE IV

Liposome Attachment Assay for Vitronectin Receptor (Vn-R)

This assay was performed with minor modifications, according to the method of Pytela et al., Methods Enzymol. 144:475 (1987), incorporated herein by reference. Briefly, 1:4 mixture of labelled and unlabelled phosphatidylcholine (PC) liposomes was dissolved under nitrogen and diluted with an equal volume of purified receptor (purified as described in Example II) to achieve a fixed predetermined receptor-liposome concentration ratio. This mixture was then dialyzed overnight at 4°C in phosphate buffered saline (PBS) containing 1 mM Ca^{2+} . An aliquot of the dialyzed sample was counted to assess radioactive content; the receptor-liposome mixture was then diluted to obtain a set radioactivity per unit volume.

Microtiter plates were coated with 10 μg of vitronectin. Non-specific sites were blocked for 2 hours at 37°C in PBS containing 5 mg/ml BSA and 1 mM each of CaCl_2 and MgCl_2 . The plates were then rinsed twice with PBS containing 1 mM Ca^{+2} and Mg^{+2} , and 100 μl of the liposome-receptor mixture was added to each well. If necessary, peptides were added before this step in a 1-10% dilution. The plates were then incubated at 4°C for 24 hours. The following day, the liquid in each well was aspirated and the plates were washed twice with PBS containing 1 mM Ca^{+2} and Mg^{+2} . Finally, 100 μl of 2% SDS was added, the plates were shaken for 10-15 minutes, and the supernatants were collected, vortexed, and subjected to liquid scintillation counting. This procedure typically yielded ca. 1000 total and 100 non-specific counts per well.

EXAMPLE VPlatelet Aggregation and Potencies of
Hydrophobically Enhanced RGD Peptides

5 Platelet aggregation was assessed using the method of
Born, Nature 194:927-929 (1962), incorporated herein by
reference. Briefly, the change in light transmission was
measured through a stirred suspension of platelets in an
aggregometer (Model 400 VS, Chrono-Log, Havertown, PA,
10 USA). Studies employing ADP were performed with platelet-
rich plasma (PRP), which was obtained by low-speed
centrifugation (200 x g for 10 min.) of whole blood freshly
drawn into trisodium citrate (at a final concentration of
11 mM). In studies using thrombin, the PRP was gel-
15 filtered on Sepharose 2B in divalent ion-free Tyrode's
solution containing 2% BSA. For all studies, the reference
standard was platelet-poor plasma, which was obtained by
centrifuging PRP at 1000 x g for 5 min.

20 All aggregation studies were performed at 37°C with a
constantly stirred suspension of 3×10^8 platelets/ml.
(Platelet count was determined with the aid of a
hemacytometer.) Peptides and stimulants were added to
these suspensions in 1% dilutions. The PRP and gel-
25 filtered platelets were used within three hours from the
time of blood collection.

Peptide anti-aggregation potencies were determined
from dose-responsive curves for the inhibition of the
30 maximum aggregation responses stimulated by physiologic
doses of ADP (10 μ M) and thrombin (2 U/ml). The 50%
inhibitory concentration of each peptide (IC_{50}) was
determined by regression analysis of these curves.

35 In an independent study, a slightly modified
aggregation assay was used. The modified method was as
follows and the results are shown in Table II. Platelet

aggregation assays were performed in human platelet rich plasma (PRP). Fifty milliliters of whole human blood (9 parts) was drawn on 3.6% sodium citrate (1 part) from a donor who had not taken aspirin or related medications for at least two weeks. The blood was centrifuged at 160 x g for 10 minutes at 22°C and allowed to stand for 5 minutes after which the PRP was decanted. Platelet poor plasma (PPP) was isolated from the remaining blood after centrifugation at 2000 x g for 25 minutes. The platelet count of the PRP was diluted to about 300,000 platelets per microliter with PPP.

A 225 μ l aliquot of PRP plus 25 μ l of either a dilution of the test inhibitor sample or a control (PBS) was incubated for 5 minutes in a Chrono-log Whole Blood Aggregometer at 37°C. An aggregating agent (collagen, 1 μ g/ml; U46619, 100 ng/ml; or ADP, 17 μ M) was added and the transmission was recorded.

The hydrophobically enhanced RGD peptides have been grouped into four distinguishable classes for systematic comparison. They are (1) cyclic RGD peptides which vary the size and hydrophobicity of the moiety at the position immediately following the Asp residue in the tripeptide RGD (the first and last position as depicted in Table I may vary by substitution with Arg); (2) cyclic RGD peptides which vary the size and hydrophobicity of the bridging structure for cyclization; (3) cyclic RGD peptides which vary the size and hydrophobicity of both the bridging structure and the residue immediately following the Asp residue in the tripeptide RGD and (4) cyclic RGD peptides which fall into one of the above three classes and also vary the charge at the residue adjacent to the hydrophobic moiety. Other RGD peptides, both linear and cyclized, are included in Table I for comparison. Underlining indicates a bridge between the first and last residue included in the underlined portion.

As shown in Table I, each class of cyclized, hydrophobically enhanced RGD peptide analogue demonstrated inhibitory effects on platelets stimulated with thrombin or ADP. Eight analogues had inhibitory potencies (IC_{50}) less than or approximately equal to $10 \mu M$ against thrombin-stimulated platelet aggregation while as many as twenty-two demonstrated inhibitory potencies in this range for the ADP-stimulated response. For example, the inclusion of hydrophobic residues phenylalanine (F) and tryptophan (W) in the "X" position of template structures GPenGRGD-X-PCA and GPenGHRGD-X-RCA imparted greater anti-aggregation inhibitory potency relative to GPenGRGDSPCA and GPenGHRGDLRCA. This effect was further enhanced by other non-natural hydrophobic structures, such as para-chloro-phenylalanine (PheCl), and para-methyl-tyrosine (TyrMe) O-n-hexyl-tyrosine, 3,5 -diiodo-tyrosine, O-n-butyl-tyrosine, p-nitro-phenylalanine and the like in the same position. The inclusion of positively charged moieties such as arginine (R) or lysine (L) in the "X" positions of XPen-GRGDSPCA or X-PenGHRGDLRCA also increased anti-aggregation potency. Moreover, the inclusion of a positively charged moiety outside the cyclic structure also yielded peptides with high potency.

Organic mimic bridging structures were substituted for penicillamine and Pmp in the "X" position of the template structure G-X-GHRGDLRCA. When substituted alone, tert-butyl-Pmp and amino-Pmp lessened peptide anti-aggregation potency. On the other hand, peptide derivatives containing these moieties and an N-terminal R significantly outperformed the previously disclosed cyclic RGD structures GPenGHRGDLRCA and PmpGHRGDLRCA, in platelet aggregation assays. Finally, replacement of 1-Pen in G(1-Pen)GHRGDLRCA by the d-form of penicillamine lowered anti-aggregation potency by 2-fold.

The modifications described above have resulted in

inhibitory potencies 10 to 250-fold more potent than the prototype GRGDSP linear peptide and 2 to 5-fold more potent than the initial conformationally restrained cyclic peptide GPenGRGDSPCA. The results from the independent study, 5 shown in Table II, further corroborate these conclusions.

TABLE I

Potencies of Hydrophobically Enhanced RGD Peptide
Analogues Against Platelet Aggregation and Receptor Binding

5	Peptide	Thrombin Stimulated Aggregation	ADP-Stimulated Aggregation	F _{NR} Binding	V _{NR} Binding	IIb/IIIa Binding
10	GRGDSF		135±15	0.032±0.006(4)*	0.70	50
	GPENGGRGDSPCA	28	27.5	0.015	0.15	0.29
	GPENGGRGDTPCA		22.3	.063	0.033	
	GPENGGRGDLPCA		19.3	0.96	0.073	
15	GPENGGRGDFPCA		3.5	.084	2.7	4.1
	GPENGGRGDWPCA	0.53	1.6	0.13	12.7	
	RPENGGRGDWPCR		0.65	0.014	4.2	0.098
20	GPENGHRGDLRCA	18	15	10	1.2	10
	RPENGHRGDLRCR	3.2	10.3	5.9	37.1	6.2
	RPENGHRGDWRCR	1.2	1.3	7.3	6.9	0.80
	RPENGHRGD(Cha)RCR		4.1	3.9	10	
	RPENGHRGD(1-napa)RCR		29	10		
25	PmpGHRGDLRCA	6.3	7.2	0.34	10	
	G(am-Pmp)GHRGDLRCA		83±23(2)	3.4		
	(t-but-Pmp)GHRGDLRCA		120	1.1		
	(t-but-am-Pmp)GHRGDLRPenA		89			
	G(dPen)GHRGDLRCA		6.28±0.7(2)	1.5	5.3	1.4

Peptide	Thrombin Stimulated Aggregation	ADP-Stimulated Aggregation	F _{NR} Binding	V _{NR} Binding	IIb/IIIa Binding
5 R(am-Pmp)GHRGDWRCR-1		1.4	1.7	5.9	0.06
R(am-Pmp)GHRGDWRCR-2 ^a		1.4	1.7		
R(am-Pmp)GHRGD(TyrMe)RCR		0.45	2.1	1.0	0.018
R(am-Pmp)GHRGD(PheCl)RCR		0.65	1.2	10	0.02
R(am-Pmp)RGD(Cha)CR		<200*	0.35		
10 R(am-Pmp)GHRGDLRCR-1	1.3	1.5	1.1	6.7	0.4
R(am-Pmp)GHRGDLRCR-2 ^a	1.3	1.5	2.9		
R(t-but-am-Pmp)GHRGDLRCR-1		1.9	2.2		
R(t-but-am-Pmp)GHRGDLRCR-2 ^a		1.9	2.2		
R(am-Pmp)GRGDWPCR		1.2	0.05	3.7	0.06
15 RPMCGHRGD(O-n-hexyl-Tyr)RCR		0.96	2.0	0.84	0.025
RPMCGhrqd(3,5-diiodo-Tyr)RCR		1.10	6.1	0.9	0.013
RPMCGHRGD(Hpa)RCR		1.15	1.81	4.2	0.02
RPMCGHRGD(2-Nal)RCR		1.60	4.2	10	0.06
RPMCGHRGD(O-n-butyl-Tyr)RCR		0.35	6.60	2.20	0.028
RPMCGHRGD(p-nitro-Phe)RCR		0.38	5.74	6.05	0.023
20 RPMCGHRGDVRCR		0.69	2.30	2.00	0.15
RPMCGHRGDFRCR		0.62	8.70	10.00	0.043
RPMCGHRGDYRCR		1.00	10.00	7.85	0.153
RPMCRHRGD(O-Me-Tyr)RCR		0.44	7.72	6.20	0.068
25 RPMCGHRGD(Phq)RCR		0.68	1.23	0.79	0.023
RPMCGHRGD(O-Me-Tyr)RCR		0.83	6.40	9.90	0.06
FPmCGHRGD(O-Me-Tyr)RCR		0.73	8.70	6.04	0.07
R(dPen)GHRGD(p-Iodo-Phe)RCR		0.78	4.37	10.00	0.023
KPMCGHRGD(O-Me-Tyr)RCR		0.83	8.60	3.90	0.029
30 RPMCGHRGD(O-Me-Tyr)OCR		0.91	8.40	9.30	0.007
HPmCGHRGD(O-Me-Tyr)RCR		0.94	8.30	8.40	0.079
RPMCGHRGD(p-amino-Phe)RCR		0.99	9.01	10.00	0.34
RPMENHRGD(O-Me-Tyr)RCR		0.99	4.70	6.90	0.098
GPmCGHRGD(O-Me-Tyr)RCR		1.10	10.00	10.00	0.104

Peptide	Thrombin Stimulated Aggregation	ADP-Stimulated Aggregation	FnR Binding	VnR Binding	IIb/IIIa Binding
5 <u>RPmcGHRGD(O-Me-Tyr)KCR</u>		1.10	10.00	10.00	0.047
<u>GPenIARGDWNCA</u>		1.20	0.71	0.041	0.01
<u>TPmcGHRGD(O-Me-Tyr)RCR</u>		1.25	10.00	10.00	0.052
<u>RPmcGHRGD(p-nitro-Phe)KCR</u>		1.40	3.10	10.00	0.10
<u>RPmcGHRGD(p-iodo-Phe)KCR</u>		1.50	5.50	8.00	0.063
10 <u>AcPmcGHRGD(O-Me-Tyr)RC-NH₂</u>		1.61	9.70	10.00	0.40
<u>DPmcGHRGD(O-Me-Tyr)RCR</u>		1.75	10.00	8.85	0.22
<u>AcCIPRGD(O-Me-Tyr)RC-NH₂</u>		0.17	4.28	5.75	0.003
<u>ACCNPRGD(O-Me-Tyr)RC-NH₂</u>		0.22	8.20	4.70	0.029
15 <u>Ac(dPen)IPRGD(O-Me-Tyr)RC-NH₂</u>		0.28	4.31	4.32	0.0023
<u>Ac(dPen)NPRGD(O-Me-Tyr)RC-NH₂</u>		0.17	6.75	6.71	0.0010
<u>ACCNPRGD(O-n-butyl-Tyr)RC-NH₂</u>		0.10	1.53	10.00	0.006
<u>R(dPen)NPRGD(O-Me-Tyr)RCR</u>		0.12	10.00		0.0012
20 <u>GRGDSPDG</u>		32	0.05	0.10	0.25
<u>FRGDSPDG</u>		110	2.4	0.04	
<u>YRGDSPDG</u>		83	1.1	0.08	
<u>LRGDSPDG</u>		150	1.2	0.2	
<u>dsRGDSPDG</u>		54	.075	0.07	
25 <u>VRGDSPDG</u>		92	0.04	0.24	0.27
<u>GPenGRGDRPCA</u>		22.3	1.3	0.47	
<u>GPenLRGDTPCA</u>		86	1.9	0.25	
<u>GPenVRGDSPCA</u>		45			
<u>GPenYRGDSPCA</u>		47			
30 <u>GPenLRGDSPCA</u>	308	415	0.54	0.26	0.48
<u>GPenLRGDSRCA</u>	49	41	1.1	1.35	
<u>GPenGRGDSFCA</u>		115		0.02	
<u>GPenFRGDSFCA</u>		>200	0.33	0.05	3.4
<u>GPenGRGDTPCR</u>		22.0	0.26	0.01	
35 <u>RPenGRGDTPCA</u>	3.1	7.0	0.08	0.03	

Peptide	Thrombin Stimulated Aggregation	ADP-Stimulated Aggregation	FnR Binding	VnR Binding	IIb/IIIa Binding
5 <u>KPenGRGDTPCA</u>		10.3	0.03		
<u>RPenGRGDTPCR</u>	2.0	6.3	.027	0.015	
<u>RPenGRGDTPCR</u>		7.6	0.49	0.01	
<u>RPenGRGDSRCA</u>	12				
<u>RPenGRGDLRCA</u>	13				
<u>GPenGRGDTPCA</u>	17	11.3			
<u>R₂PenGRGDTPCA</u>	2.7	8.2	2.9		
<u>RPenGRGDTPCR</u>		8.8			
<u>LPenGRGDLRCA</u>		10.5			
<u>PmpGRGDSPCA</u>		45	4.9		
<u>PmpGRGDTPCA</u>		85	.023	0.10	0.58
<u>PmpGRGDLRPenA</u>		5.8	.086	0.05	
<u>(t-butylamino-Pmp) GHRGDLRCA</u>		130	.078	5.2	0.55
<u>R(AminoPmp)GRGDTPCA</u>			1.5		
<u>GHRGDLRDASG</u>			0.03		
<u>RKGHRGDLRDR</u>		7.5	4.9		
<u>R(orn)GHRGDLRDASG</u>		75	1.3		
<u>R(orn)GHRGDLRDR</u>		24	22		
<u>R(orn)GHRGDLRER</u>		>200			
<u>GHRGDLRPaSA-NH₂</u>		44	1.7		
25 <u>R₉GDS</u>		4.8	3.2		
		28	0.64		

32

*concentrations unconfirmed by amino acid composition analysis

30 ^a Represents second peak after HPLC purification of synthesized peptides using a racemic am-Pmp mixture.

TABLE II
In Vitro Integrin ELISA Results

Code	IC50 (μ M) Human Platelet Aggregation	IC50 (nM)				Sequence
		Fg/II _b III _a	Vn/VnR	vWF/II _b III _a	Fn/FnR	
10	8X	0.22	4700	14.8	8200	Ac-CNPRGD(Y-OMe)RCNH ₂
	11J	3.4	48	6.4	6200	Ac-CNPRGD(Y-OMe)EC-NH ₂
	11K	4.5	30	9.3	840	Ac-CNPRGD(Y-OMe)AC-NH ₂
	11L	7.3	1818	640	>10000	Ac-CNPRGD(Y-OMe)(dR)C-NH ₂
	11P	4.9	63.5	9.9	440	Ac-CNPRGD(Y-OMe)QC-NH ₂
15	11Q	0.79	20000	60.2	5600	Ac-CNPKGD(Y-OMe)RC-NH ₂
	11R	1.0	705	89.9	1560	Ac-CNPRGD(Y-OMe)KC-NH ₂
	11S	7.3	891	27.1	440	Ac-CNPRGD(Y-OMe)(Cit)C-NH ₂
	11V	7.6	108	18.6	(not tested)	Ac-CNPRGD(Y-OMe)(Nle)C-NH ₂
20	2G	14				GPENGHRGDLRCA
	2H	20				GRGDSPDG
	4Q	3.3		86.6		RPENGHRGDWRCR
	50	0.56		15.7		RPmcGHRGD(Y-OMe)RCR
	6Z	58				GPENRARGDNPACA
25	7B	0.70		11.8		RPmcGHRGD(p-I-F)RCR
	7T	0.38		29		RPmcGHRGD(p-NO ₂ -F)RCR
	10R	0.17				AcCIPRGDY-OMeRCNH ₂
	10X	0.17				Ac(dPen)NPRGDY-OMeRCNH ₂

EXAMPLE VI

Peptide Receptor Selectivity

In parallel studies with the platelet aggregation
5 experiments (described in Example V), the apparent
affinities of peptides for GP IIb/IIIa, fibronectin and
vitronectin receptors were determined. Receptor-binding
assays (as described in Examples III and IV) with purified
receptors, were used to assess the abilities of the
10 peptides to displace the binding of receptors to their
receptor-specific ligands.

Shown in Table I, as a comparison with platelet
inhibitory potencies, are the relative affinities of each
15 peptide for the receptors shown.

The binding data are again represented as the 50%
inhibitory concentration of each peptide (IC_{50} s) and were
determined as described in Example V for the dose-response
20 curves for the inhibition of platelet aggregation. IC_{50} s
for FnR were determined by ELISA (Example III). Those for
VnR and GP IIb/IIIa were determined either by ELISA or
liposome attachment assay (Example IV). The GRGDSP
prototype peptide is used as a reference for comparison
25 between assays for an individual peptide.

Table II provides the receptor binding data as a
comparison with the platelet inhibitory potencies shown
therein. This data is also represented as the 50%
30 inhibitory concentration of each peptide. The values for
all receptor assays were determined by ELISA as described
in Example III. The results shown in Table II further
corroborate the conclusions drawn from Table I, namely,
that the anti-aggregation potencies are parallel by their
35 relative affinity for the IIb/IIIa receptor.

EXAMPLE VIIEfficacy Against Electrically Induced
Canine Coronary Thrombosis

5 1. Surgical Preparation and Instrumentation

Male mongrel dogs weighing 14 to 20 kg were selected based on proper aggregation of their platelets in response to arachidonic acid, collagen, and adenosine disphosphate
10 (ADP) and based on similar weights and hemodynamic properties.

Before surgery, the animals were anesthetized with sodium pentobarbital (30 mg/kg, i.v.) and then intubated
15 and ventilated on room air with positive pressure using a respirator (Harvard Apparatus, S. Natick, MA) at a volume of 30 ml/kg and a frequency of 12 breaths/min. Surgery, performed under aseptic conditions, was begun with the placement of cannulae into the left carotid artery and
20 jugular vein for monitoring arterial blood pressure (Statham P23 pressure transducer, Gould, Inc., Cardiovascular Products, Oxnard, CA) and administering intravenous fluids.

25 The heart was exposed via a left thoracotomy through the 5th intercostal space. A 2-cm segment of the left circumflex coronary artery (LCCA) was isolated from surrounding tissue by blunt dissection. This artery was instrumented from proximal to distal with an
30 electromagnetic flow probe (Model 501, Carolina Medical Electronics, Inc., King, NC), intra-coronary electrode, and screw occluder (see Figure 1). The intra-coronary electrode was constructed by attaching a 25-gauge hypodermic needle tip to a 30-gauge Teflon-insulated
35 silver-coated copper wire. The mechanical occluder was constructed of stainless steel in a C shape with a Teflon screw (2 mm diameter) on top. It was adjusted to control

vessel circumference and decrease the reactive hyperemic flow due to a 10-sec (full) occlusion by 50-70% without affecting basal coronary blood flow (Figure 1). A monopolar epicardial electrode was sutured to the surface of the ventricle in the region of LCCA distribution to monitor ischemic changes in the electrocardiogram (ECG). The left atrium was cannulated with polyethylene tubing for administration of the peptide. Continuous recordings of blood pressure, limb lead II ECG, epicardial electrogram, and mean and phasic LCCA blood flow were obtained on a Model 7 polygraph (Grass Instrument Co., Quincy, MA).

2. Induction of coronary thrombosis

One hour after the completion of surgery, a 100 micro-amp continuous anodal direct current delivered from a 9 volt nickel-cadmium battery was applied to the intimal surface of the LCCA. The anode of the battery was in series with a 250,000 ohm potentiometer and the intraluminal coronary artery electrode. The electric circuit was completed by placing the cathode in a subcutaneous site. Electrical stimulation was applied for three hours. At the conclusion of each experiment, the heart was fibrillated electrically and quickly removed, and the LCCA was dissected free as far as possible to verify the position of the implanted anodal electrode.

For all ex vivo studies (see Figure 3), control aggregation values were standardized to the percentages of light transmission observed in PRP and PPP samples (0% and 100%, respectively).

3. Peptide Administration

Animals were randomly assigned to two treatment groups: vehicle control (i.e., normal saline) or RGD peptides at various concentrations. Peptides were

administered intra-atrially in both bolus and continuous injections. Each bolus injection consisted of 0.5 to 10 mg/kg and was administered 15 minutes before application of the current. A continuous infusion of the same peptide was then started immediately after completion of this initial injection. Animals received a 25 μ g/kg/min to 200 μ g/kg/min infusion. The anti-thrombotic effects of the peptides were monitored until 30 minutes after the occurrence of a persistent, occlusive thrombus or five hours after the initiation of electrical stimulation (whichever resulted first). If an occlusive thrombus had not developed within four hours after the initiation of current, the peptide infusion was stopped.

4. Platelet Studies

Platelet counts and ex vivo aggregation studies were performed one hour before and 1, 3, and 5 hours after application of anodal direct current. Samples of arterial blood were drawn into plastic syringes containing 3.8% trisodium citrate (giving a 1:10 final dilution) and platelet aggregation determined as described in Example V.

The inhibitory potencies of peptide analogues 2 G (GPenGHRGDLRCA), 4Q (RPenGHRGDWRCR), and 4R (RPenGRGDWPCR), as well as the generic analogue GRGDSP, were determined against canine platelet aggregation stimulated maximally by 10 μ M ADP, 0.65 mM arachidonic acid (AA), or 9.6 micrograms/ml collagen. Epinephrine (550 nM) was used to prime platelets before stimulation with arachidonic acid. Peptides were added in 1% dilutions to the PRP solutions. The relative anti-aggregation potencies of all injected peptides and the generic analog GRGDSP were determined at the 1 hour before current time point, with dose-response analysis (see Figure 2). The peptide concentrations causing 50% inhibition of maximal activation (IC_{50} s) were derived by linear regression of these dose-response curves.

For computation of these inhibitory potencies, control values (i.e., in the absence of peptide) were considered as 100% of maximum.

5 As shown, analogues 4Q and 4R exhibited superior potencies, inhibiting aggregation by 50% at 1.5 - 5 μ m. Analogue 2G was slightly less potent, with IC_{50} s of 15 - 30 μ m, whereas GRGDSP inhibited all three responses by 50% at ca. 130 micromolar. Notably, the potency order (4Q = 4R > 10 2G > GRGDSP) and IC_{50} s of these peptides against these responses were the same as those observed for their inhibition of ADP-, collagen-, and arachidonic acid-stimulated aggregation of human platelets.

15 Platelet aggregation was also determined ex vivo at one, three, and five hours after current application. For these studies, arachidonic acid, or collagen was again used to stimulate the platelets.

20 The average aggregation values determined in these studies for all of the peptide treatments are depicted in Figure 3. (In these histograms, 0% and 100% aggregation represent the extent of light transmission through PRP and PPP, respectively, before the addition of stimulant.) 25 Analogue 2G, when injected at 10 mg/kg, substantially inhibited ADP-stimulated aggregation but only partially inhibited the AA- and collagen-stimulated responses (43 - 70% and 12 - 50%, respectively, relative to control levels) at all three time points (Figure 3a). As shown in Figure 30 3b and 3c, the ex vivo anti-aggregation effects of analogues 4Q and 4R were far superior. A 5 mg/kg injection of analogue 4Q made the platelets completely unresponsive to stimulation with ADP and AA at all three time points and caused near-maximal inhibition of their activation by 35 collagen. (Here, the absence of a coded bar indicates the absence of the corresponding response.) As shown in Figure 3b, the effects of this same peptide at the same injection

were more pronounced when platelet count was low, i.e., ca. one-third of normal (104,000/ml vs. 361,000/ml). At a higher injected concentration (10 mg/kg), analogue 4Q prevented platelet aggregation by all three stimuli at one-
5 and three-hour time points. At the five-hour point, platelet responsiveness was slightly improved. The control responses in these studies were 70 - 80% of maximum. As shown in Figure 3c, analogue 4R, at 3 mg/kg, exerted an apparent time-dependent effect on platelet responsiveness
10 in that aggregation was reduced only 20 - 58% at one hour but 75 - 100% at three and five hours relative to control levels. Finally, a 10 mg/kg injection of this analogue caused the platelets to be unresponsive to all modes of stimulation at all time points. Here the control responses
15 were 55 - 80% of maximum.

5. Peptide Effects on Coronary Blood Flow and Thrombosis

Coronary thrombosis was quantified as the time to full
20 occlusion. The effects of the various peptide treatments on coronary thrombosis is illustrated in Figure 4. In the control situation (saline injections), a full occlusion was observed in slightly more than two hours. Analogue 2G, even at 10 mg/kg, did not significantly influence this
25 frequency. Analogue 4Q at 5 mg/kg significantly prolonged the time to occlusion and at 10 mg/kg completely prevented occlusion for the full five-hour experimental period. Moreover, at the low dose (5 mg/kg), analogue 4Q was able to prevent thrombus formation in animals whose circulating
30 platelet levels were one-third of normal. Analogue 4R at 10 mg/kg prevented occlusion throughout the duration of the study but at 3 mg/kg was ineffective.

In these studies, the degree of anti-thrombotic
35 efficacy appeared to coincide with the anti-aggregation potency described above. For example, analogues 4Q and 4R, which were superior inhibitors of in vitro aggregation,

also exerted a considerably greater in vivo protective effect than analogue 2G at the same injected concentration. Moreover, these peptides were able to prevent full occlusion only when they completely prevented platelet stimulation by all of the agonists (at 10 mg/kg). However, analogue 4Q (at 5 mg/kg) completely or near-maximally blocked all aggregation responses but could merely prolong coronary occlusion. In addition, analogue 4R at 3 mg/kg blocked aggregation responses by 72 - 100% at the three- and five-hour points, yet at these times an occlusive thrombus had fully developed. Finally, these peptides could completely prevent occlusion in this model only at injected concentrations equivalent to 20- to 50-fold greater than their IC_{50} s against in vitro aggregation.

6. Hemodynamic Responses

Bleeding time was quantified at 1 hour before and one, three, and five hours after administration of the peptide. This was done by making a small (5 mm long and 1.5 mm deep) incision in the tongue and subsequently absorbing the exuded blood at this site every 15 seconds with a piece of Whatman filter paper until bleeding stopped. Platelet counts were determined with a Haema Count MK-4/HC platelet counting system.

It is important to note that these apparently excessive peptide concentrations did not exert any significant effects on template bleeding time, platelet counts, or on the main hemodynamic parameters (heart rate and blood pressure), which remained essentially unchanged and similar to baseline values throughout the experimental periods (Figure 5). In cases where peptide treatment did not prevent occlusion, at certain times these parameters were not determined (ND in Table II), as experiments were terminated 30 min. after circumflex coronary artery blood flow had ceased due to occlusive thrombus formation.

EXAMPLE VIII

Anti-Thrombotic Properties of Hydrophobically
Enhanced RGD Peptides in Prosthetic Arterial Grafts

- 5 Adult male baboons (weighing 16 to 25 kg) were used in these studies. These were sedated with ketamine hydrochloride (200 to 250 mg intramuscular injection) and maintained under anesthesia with sodium pentobarbital (50 to 75 mg administered intravenously as necessary).
- 10 Twenty-four hours before the ex vivo shunt was established, platelets were isolated from 500 ml of blood from the test animal and labelled with ca. 500 microcuries of indium-111 oxine (Medi+Physics, Emeryville, CA), which irreversibly and specifically binds to platelets with an
- 15 efficiency of 50%. Immediately after labelling, these platelets were then injected back into the animal and allowed to circulate for 24 hours. Immediately before the start of the study, fibrinogen that had been isolated from the animal and labelled with iodine-131 (DuPont Nuclear,
- 20 Boston, MA) was also injected back into the animal. Also at this time, baseline determinations of the clotting and template bleeding times were made, and blood samples were drawn for hematology studies.
- 25 To establish the ex vivo shunt, the femoral artery and vein were percutaneously cannulated with introducer catheters (KMA Inc., Mansfield, MA). The catheters were then connected to medical-grade, heparin-coated silastic tubing (2.59 mm internal diameter, (Extracorporeal Medical
- 30 Specialties, Inc., King of Prussia, PA). An electromagnetic flow probe was then inserted into the tubing by varying the resistance imparted by a partially occluding screw clamp that was distal to the probe. Finally, a 5 cm-long test segment of a 4 mm (internal
- 35 diameter) vascular graft was inserted at the apex of the circuit. The graft used in these studies was Gore-tex

(expanded polytetrafluoroethylene, (W.L. Gore and Associates, Inc., Flagstaff, Az). An electromagnetic flow probe was then inserted into the tubing circuit to measure blood flow, which was maintained at 100 ml/min. by varying the resistance imported by a partially occluding screw clamp that was distal to the probe. Finally, a 5 cm-long test segment of a 4 mm (internal diameter) vascular graft was inserted at the apex of the circuit. The graft used in these studies was Gore-tex (expanded polytetrafluoroethylene, W.L. Gore and Associates, Inc., Flagstaff, AZ).

Platelet deposition onto the grafts was monitored by dynamic scanning with a gamma camera (Sigma 400, Ohio Nuclear, Inc., Sohon, OH), which detects the gamma radiation emitted by the ¹¹¹indium-labelled platelets. Once the circuit was in place, the animal was placed under this camera, and blood flow was initiated. Scans were then taken at the rate of one frame per two minutes for two hours. The data from these scans were collected on a dedicated Digital MDA computer (Maynard, MA). The scans were corrected for graft size, isotope dose and decay, circulating platelet activity and background, and the surface areas of the grafts.

At one- and two-hour time points, template bleeding times were measured, and blood samples were drawn to assess the hematology aggregation studies. Platelet aggregation studies were performed as described in Example V using ADP as the stimulus.

After a second, identical shunt was attached to the animal, the anti-platelet peptide GPenGHRGDLRCA was administered as an intravenous (IV) injection. A second series of scans was then obtained to ascertain the effect of the peptide on the platelet uptake pattern of the graft.

Upon the completion of each study, each shunt was flushed with lactated Ringer's solution, and each graft was then removed. Sections of these grafts were subjected to liquid scintillation counting to determine their content of residual ^{131}I -iodine-fibrinogen and ^{111}In -indium-platelets. The catheters in the femoral artery and vein were then removed, and hemostasis was achieved by compression. Finally, post-procedural blood samples were drawn, and determinations of template bleeding and clotting times were also made.

10 Three different animals were used in order to account for animal variability. Two of the three test animals displayed normal platelet uptake patterns, as determined from gamma camera images of ^{111}In -labeled platelets on the graft material. Treatments for these two animals are
15 described below.

In the first of these animals, the peptide was administered as two bolus IV injections of 10 mg/kg (160 mg per injection). The first injection was given fifteen
20 minutes before the establishment of the second shunt and the second injection was administered one hour afterward. As shown in Figure 6, these injections caused a significant reduction in both ^{111}In -platelet and ^{131}I -fibrinogen uptakes (90% and 79%, respectively). This inhibitory effect is
25 also apparent from a plot of platelet uptake rates in peptide-treated and untreated grafts over the entire time course of the studies (Figure 7). Here, the rate of ^{111}In -labelled platelet accumulation represents the counts observed in a graft piece minus those found in a background
30 section of tubing at each time point when a scan was performed.

As shown in Figure 8, peptide treatment did not lower template bleeding and clotting times. In blood samples
35 taken immediately after completion of the second shunt, white blood cell and platelet counts, however, were reduced

by 37% and 14%, respectively. Other parameters were unaffected.

For the second animal, a 10 mg/kg bolus (250 mg) of the peptide was again given IV fifteen minutes before the initiation of the second shunt. This was immediately followed by a continuous infusion of 10 mg/kg/hr that lasted for the entire two hours of the shunt (500 mg total). As shown in Figure 9, this treatment also caused significant reductions in labelled platelet and labelled fibrinogen uptakes (84% and 78%, respectively). Platelet uptake rates were again plotted in the presence and absence of the peptide (Figure 10).

In addition, platelet aggregation studies were conducted on PRP derived from the second animal. Whole blood was drawn at three time points (0, 1 and 2 hours) of both control and experimental shunts. Platelets were completely unresponsive to the peptide treatment at a maximally effective concentration of ADP ($10 \mu\text{M}$). The peptide treatment also had no effect on template bleeding time, clotting time, or on all blood cell counts (Figure 11).

25

EXAMPLE IX

Bleeding Time and Ex Vivo Platelet Aggregation Rabbit Model

1. Animal Preparation and Blood Sampling

Unanesthetized male New Zealand White rabbits (2.5-3.5 kg) were placed in a standard rabbit restrainer. Ears were shaved and a 20G teflon catheter with flowswitch (Viggo) was placed in the medial artery, flushed with saline and locked with 1 ml of heparinized saline (10 m/ml). A 22G catheter (Abbott) fitted with an injection cap (Medex) was placed in the marginal vein of the same ear. Saline or a

GP II_b/III_a receptor antagonist, at a concentration of 1 to 3 mg/ml, was infused via the venous catheter. At time 0, 41% of the dose was given as a bolus over 2 minutes. The remainder was continuously infused over the following 60 minutes. Blood samples (3.2 ml) were collected into syringes without needles via the arterial catheter at -10, -5, 10, 45 and 60 minutes. The first 0.5 ml was discarded and the following 2.7 ml was collected directly into a syringe containing 0.3 ml of 3.8% sodium citrate. The sample was divided into 1.5 ml aliquots and centrifuged at room temperature for 5 seconds at 12,000 G. The resulting platelet rich plasma (PRP) is used to measure ex-vivo platelet aggregation (XPA). At -10 and 60 minutes an additional 1.5 ml sample is drawn for an automated blood count (Baker Instruments). Catheters are flushed and locked after every sample.

2. Ex-Vivo Platelet Aggregation

300 ml of PRP was placed in a disposable glass cuvette with a stir bar. The cuvette was placed in the temperature regulated light path of a light transmittance aggregometer (Chrono-log) and equilibrated to a 37°C. Baseline transmittance was recorded for 30 seconds, after which 10 ml of ADP (1 mM) was added and the change in transmittance recorded. The maximum change from baseline (dT) was noted for each sample. The extent of inhibition of XPA that was produced by an inhibitor was calculated for each animal as follows: Mean dTs were calculated for the pre and post infusion values, and then, percent inhibition was calculated as $(1 - dt(post)/ddt(pre)) \times 100$.

3. Cutaneous Bleeding Times (CBT)

CBT was measured at -10, -5, 10, and 45 minutes on the opposite ear, using an automated incision-making instrument (Surgicutt^R, ITD). An incision (5 mm x 1 mm deep) was made

on the dorsal surface of the ear at sites not supplied by major blood vessels. Blood was blotted away with absorbent paper placed near the incision site, every 2 to 15 seconds, to a maximum of 15 minutes. Cessation of bleeding was defined as no blood forming at the incision site for 15 seconds. The range of duplicate CBT in 40 normal rabbits was 0.88 to 3.38 minutes.

4. Peripheral Blood Flow (PBF)

For the experiments summarized in Table I, PBF was monitored by observation of the condition of the blood vessels in the rabbits' ears, prior to and during the infusion. Normal flow was defined as ears that appeared pink to red, with no visible constriction of the major blood vessels. Decreased flow applied to ears that have constricted vessels resulting in cold, pallid ears for up to 40 minutes following the start of the inhibitor infusion.

In an alternative series of experiments, (Table 2) PBF was measured quantitatively with a laser dopler flow probe (Perimed). The probe was positioned securely over the vascular bed of one ear and flow monitored continuously. Each inhibitor was infused and CBT measured in the opposing ear. No arterial catheter was placed for blood sampling, consequently XPA was measured in these animals. However, the doses used were shown in previous experiments to effectively inhibit XPA.

5. Results

CBT, XPA, and observed PBF were summarized in Figure 12. The ratio of the post to pre treatment CBT was calculated for each animal by dividing the mean of the 2 post-treatment (and during infusion) samples by the mean of the pre samples. In two saline control rabbits the mean \pm

sd ration of post treatment CBT (n=10) to a mean pre-treatment CBT was 1.12 ± 0.19 .

The experiments measuring PBF by laser dopler probe are summarized in Table III. As positive control, epinephrine was infused intravenously (1 mg over 2 minutes) at 60 minutes. The resulting vasoconstriction reduced flow to near 0 flow units within 5 minutes.

The doses listed in Figure 12 and in Table III refer to the bolus portion only. There were no significant changes in any of the blood indices measured.

TABLE III

15	<u>Drug</u>	<u>Time (min.)</u>	<u>CBT (min.)</u>	<u>PBF (units)</u>
	<u>RPenGHRGDWRCR</u>	0	2.8	282
	3.75 mg/kg	10	1.5	9
		45	ND	ND
20	<u>RPmcGHRGD(Y-OMe)RCR</u>	0	2.9	392
	1.5 mg/kg	10	0.8	5
		45	1.5	360
	<u>RPmcGHRGD(Y-OMe)RCR</u>	0	2.1	318
25	1.5 mg/kg	10	3.8	0
		45	1.2	283
	<u>AccNPRGDY-OMeRCNH₂</u>	0	1.8	263
	4.5 mg/kg	10	2.2	317
30		45	3.0	293

EXAMPLE X
Additional Results

Using the method described in Example V to measure ex
5 vivo inhibition of platelet aggregation and the method
described in Example VII to measure bleeding, four
additional peptides were tested. These peptides included
11J, 11Q, 11K and 11V. Results are shown in Table IV.
While all four peptides inhibited ex vivo platelet
10 aggregation at the doses indicated, peptides 11J, 11K and
11V cause extensive prolongation of bleeding time, 11Q, in
contrast, did not affect bleeding time. These results
corroborated that it is the presence of a positive charge
in the +4 position, as possessed by 11Q, which on platelet
15 aggregation inhibiting peptides confers the characteristic
of not prolonging bleeding time.

TABLE IV

5	Code	Dose	ex vivo inhibition of platelet aggregation (%)	Pre dose		Post dose	
				bleeding time (min.)		bleeding time (min.)	
10	11J	5 Mg/Kg bolus; 120 µg/Kg/min. infusion	>98	2.1		>30	
	11K	6.8 Mg/Kg bolus; 163 µg/Kg/min. infusion	>98	2.1		>30	
	11V	8.5 Mg/Kg bolus	>98	2.0		21	
	11Q	1.1 Mg/Kg bolus; 28.5 µg/Kg/min. infusion	>95	2.1		3.5	

TABLE V
Inhibition of Platelet Aggregation

5	<u>Code</u>	<u>Formula</u>	<u>IC₅₀ (μM)</u>
	11Z	Ac <u>CSPRGD(TyrOMe)RC</u> -NH ₂	0.45
	12A	Ac <u>CAPRGD(TyrOMe)RC</u> -NH ₂	0.41
	12B	Ac <u>CLPRGD(TyrOMe)RC</u> -NH ₂	0.28
10	12C	Ac <u>CDPRGD(TyrOMe)RC</u> -NH ₂	3.0
	12D	Ac <u>CYPRGD(TyrOMe)RC</u> -NH ₂	0.22
	12E	Ac <u>CRPRGD(TyrOMe)RC</u> -NH ₂	0.34

15 As can be seen from the data in Table V, the residue lying two positions to the amino side of the RGD binding site is preferably non-acidic residue.

20 INDUSTRIAL APPLICATION

 The compositions of matter disclosed and claimed herein are industrially useful for the prevention and treatment of pathologies characterized by undesirable
25 platelet aggregation, including thrombosis, stroke and vascular graft occlusion.

Although the invention has been described with reference to the presently-preferred embodiment, it should be understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims.

WE CLAIM:

1. A composition of matter comprising a cyclic RGD
5 containing peptide having a hydrophobic moiety adjacent the
carboxy terminus of the RGD sequence.

2. A composition of matter comprising a cyclic
peptide having the sequence $X_1X_2X_3X_4GD\dot{X}_5X_6X_7$ wherein X_2 and
10 X_6 comprise moieties capable of forming a bridge, X_3 is one
or more amino acids and X_1 and X_7 are zero to 20 amino acids
and X_5 comprises a hydrophobic moiety and X_4 is a positively
charged amino acid.

15 3. The composition of matter of claim 2 wherein X_2 and
 X_6 are selected from the group consisting of Cys, Pen, Pmp,
Pmc and Pas.

4. The composition of claim 2 wherein X_3 has Pro or
20 His as the carboxy terminal residue.

5. The composition of claim 2 wherein X_1 has Gly as
the carboxy terminal residue.

25 6. The composition of matter of claim 2 wherein X_5 is
selected from the group consisting of Phe, Tyr, PheCl, ChA,
TyrMe, O-n-hexyl-Tyr, 3,5-diiodo-Tyr, Hpa, 2-Nal, O-n-
butyl-Tyr, p-nitro-Phe, Phg, p-iodo-Phe, p-amino-Phe and
Cit.

30 7. The composition of matter of claim 2 wherein X_4 is
selected from the group consisting of Arg and Lys.

8. The composition of claim 2, wherein X_5 further
comprises a positively charged moiety.

9. A composition of matter comprising an RGD-binding site containing cyclic peptide having platelet aggregation inhibiting activity without substantially prolonging bleeding time.

5 10. The composition of claim 9, further comprising a hydrophobic moiety adjacent the carboxy terminus of the RGD sequence.

10 11. The composition of claim 9, further comprising a positively charged moiety located to the carboxy terminal side of said binding site.

15 12. A composition of matter comprising an $-X_aGDX_b^*$ -binding site containing cyclic peptide wherein X_a is a positively charged amino acid, X_b is one or more amino acid or other moiety so as to confer platelet aggregation inhibiting activity and wherein $*$ is a positively charged moiety located in appropriate spatial proximity to said binding site so that upon administration of the composition
20 to an animal, bleeding time is not substantially increased over that of an untreated animal.

13. The composition of claim 2, further comprising a proline adjacent the amino terminus of X_a .

25 14. The composition of claim 2, wherein said cyclic peptide contains less than about 50 amino acid residues.

15. The composition of claim 2, wherein said cyclic peptide contains less than about 25 amino acid residues.

30 16. The composition of claims 1 or 2, wherein said cyclic peptides inhibit the binding of fibrinogen to GPIIb/IIIa more than the binding of van Willebrand Factor to GPIIb/IIIa.

17. A peptide selected from the group consisting of:

RPenGRGDWPCR

GPenGHRGDLRCA

RPenGHRGDWRCR

RPenGHRGD(ChA)RCR

PmpGHRGDLRCA

G(dPen)GHRGDLRCA

R(am-Pmp)GHRGDWRCR

R(am-Pmp)GHRGD(TyrMe)RCR

R(am-Pmp)GHRGD(PheCl)RCR

R(am-Pmp)GHRGDLRCR

R(am-Pmp)GHRGDLRCR

R(t-but-am-Pmp)GHRGDLRCR

Ac-CNPRGD(Y-OMe)RCNH₂

Ac-CNPKGD(Y-OMe)RC-NH₂

Ac-CNPRGD(O-N-Butyl-YORC-NH₂

18. The composition of matter of claim 1, 2, 9 or 17 in a physiologically acceptable carrier.

19. A method of treating or preventing thrombosis, comprising administering a therapeutically effective amount of a cyclic, RGD-containing peptide having high affinity for IIb/IIIa and low affinity for the fibronectin and vitronectin receptors.

20. A method of treating or preventing thrombosis, comprising administering a therapeutically effective amount of the composition of claim 1, 2, 9 or 17 in a physiologically acceptable carrier.

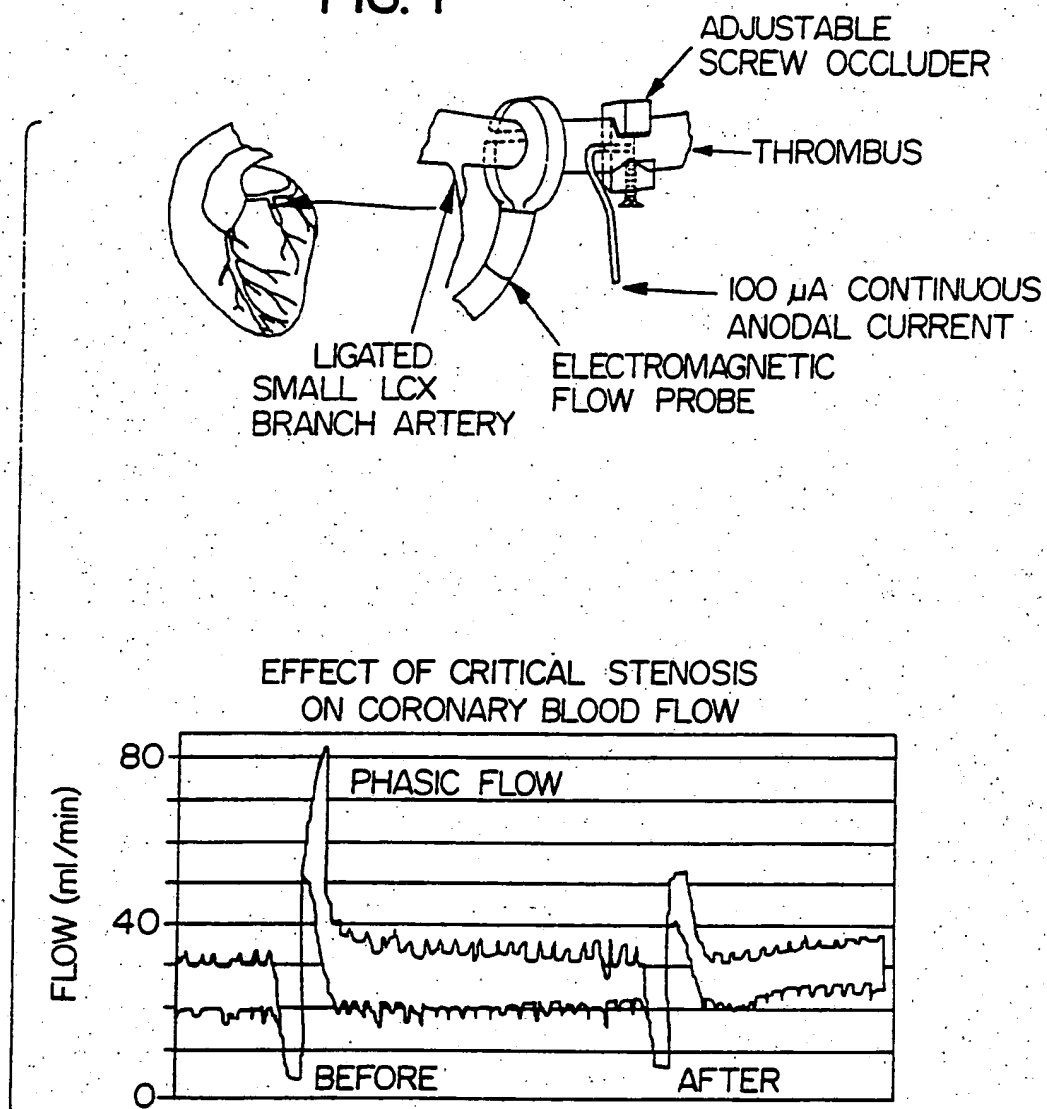
21. A method of treating or preventing vascular graft occlusion, comprising administering a therapeutically effective amount of a cyclic, RGD-containing peptide having high affinity for IIb/IIIa and low affinity for the fibronectin and vitronectin receptors.

22. A method of treating or preventing vascular graft occlusion, comprising administering a therapeutically effective amount of the composition of claim 1, 2, 9 or 17 in a physiologically acceptable carrier.

5 23. A method of treating or preventing a pathology characterized by undesirable platelet aggregation, comprising administering a therapeutically effective dose of the composition of claim 1, 2, 9 or 17, in a physiologically acceptable carrier.

10 24. The method of claim 23, wherein said pathology characterized by undesirable platelet aggregation is stroke.

FIG. 1



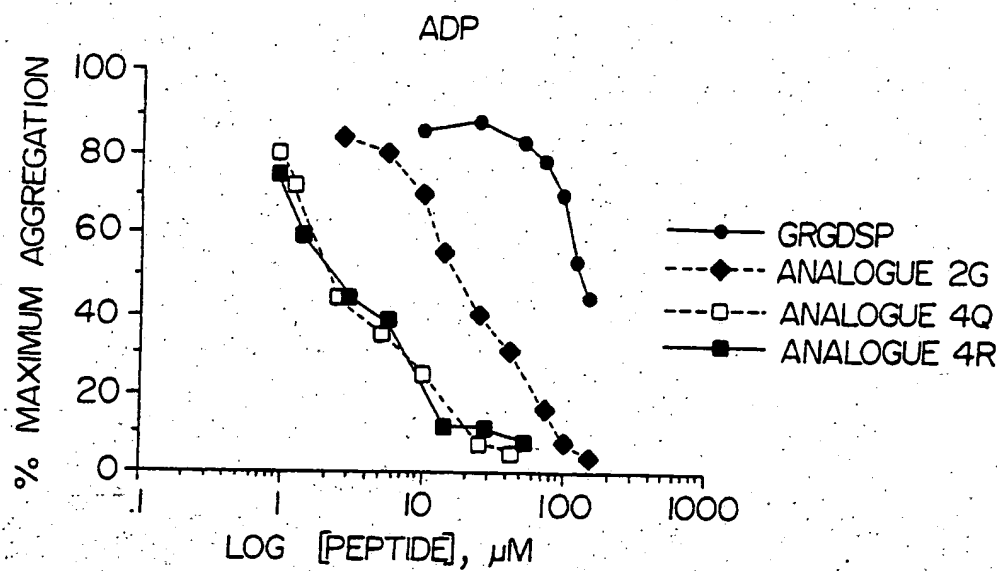


FIG. 2A

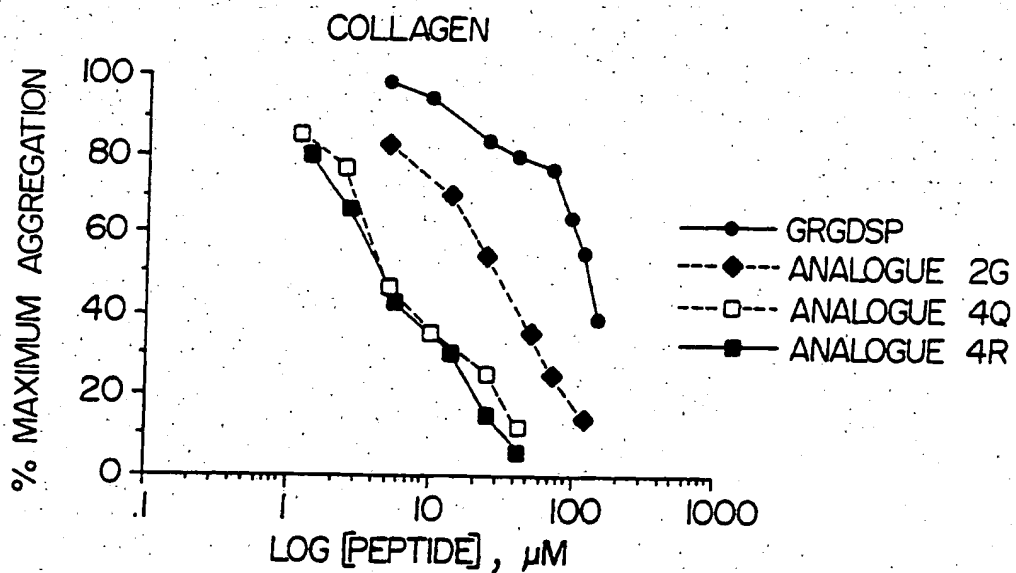


FIG. 2B

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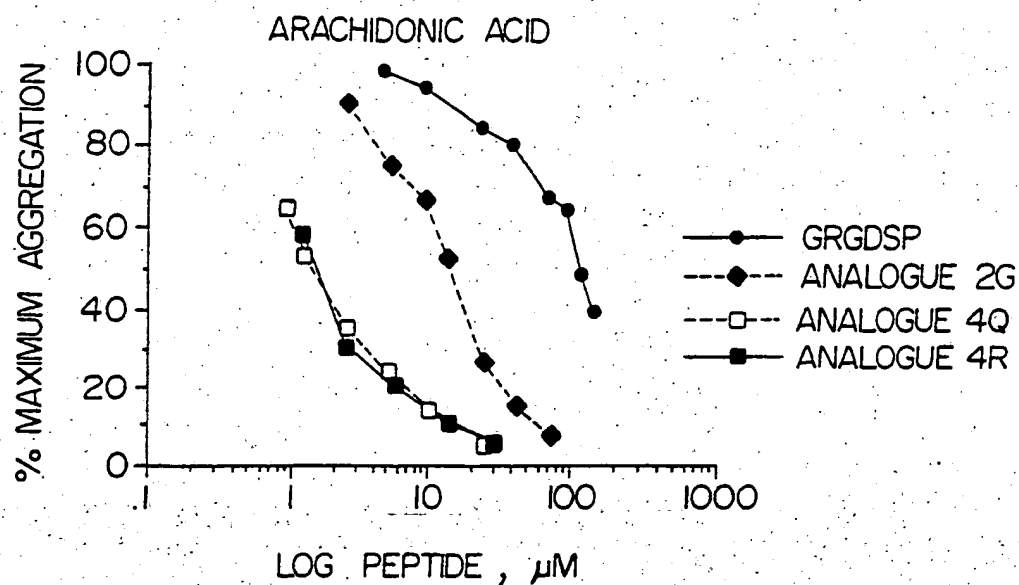


FIG. 2C

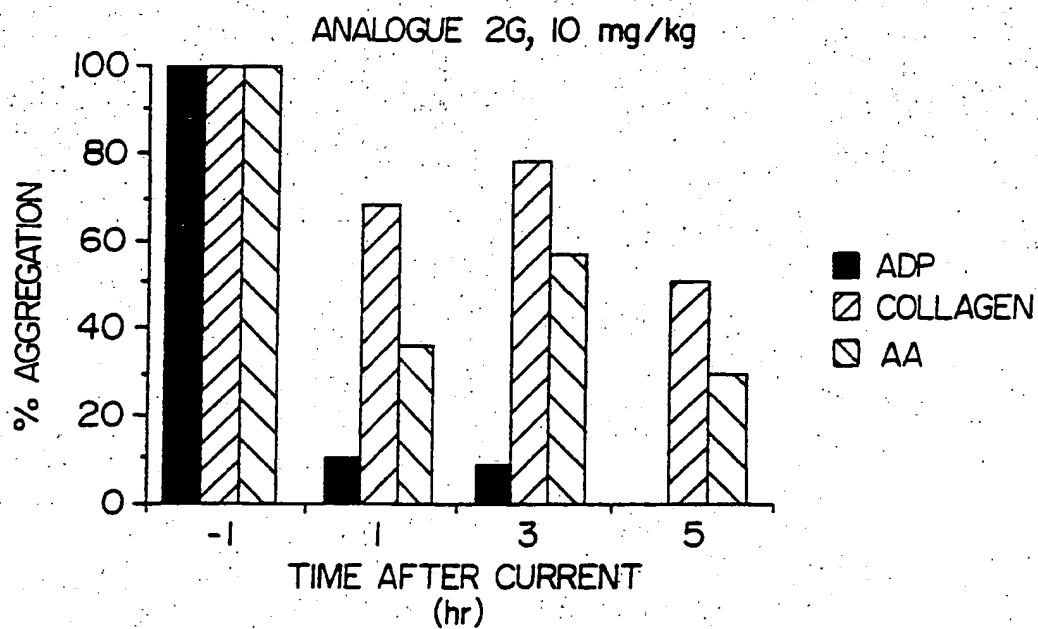


FIG. 3A

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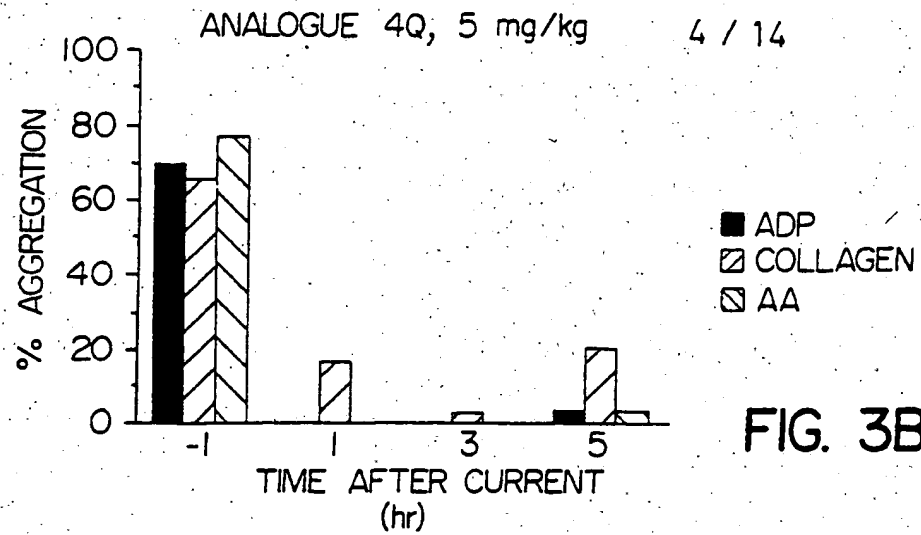


FIG. 3B

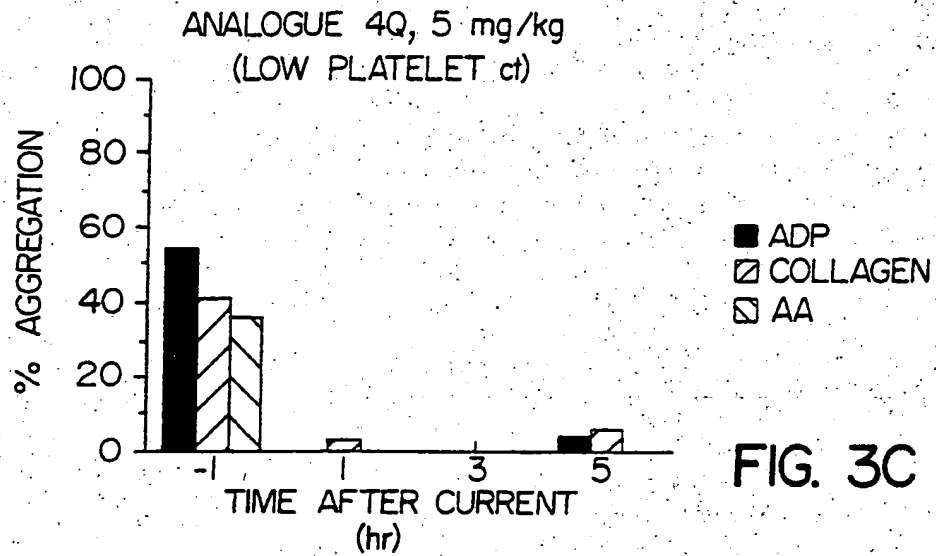


FIG. 3C

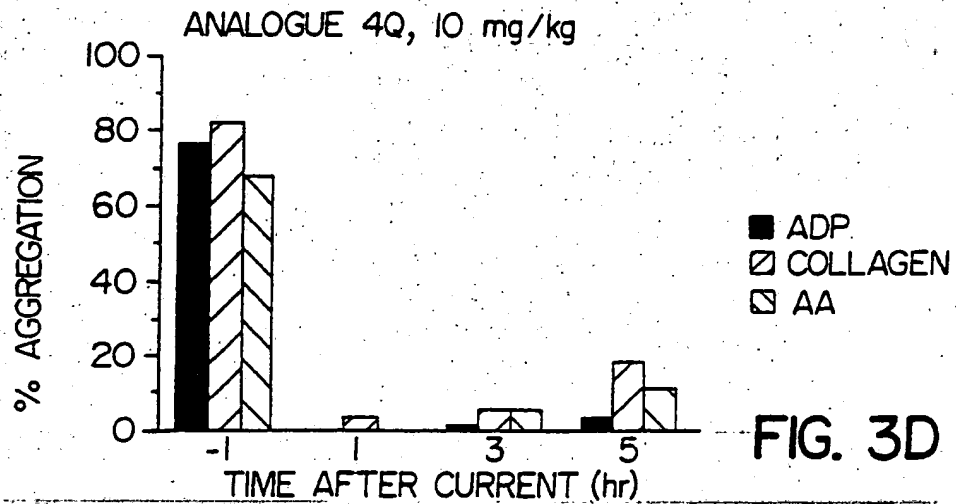


FIG. 3D

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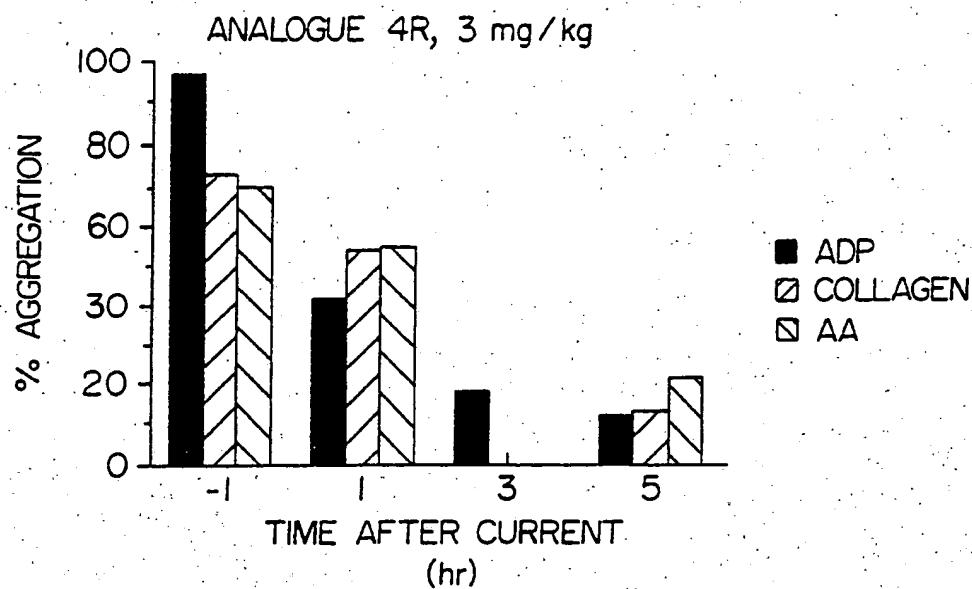


FIG. 3E

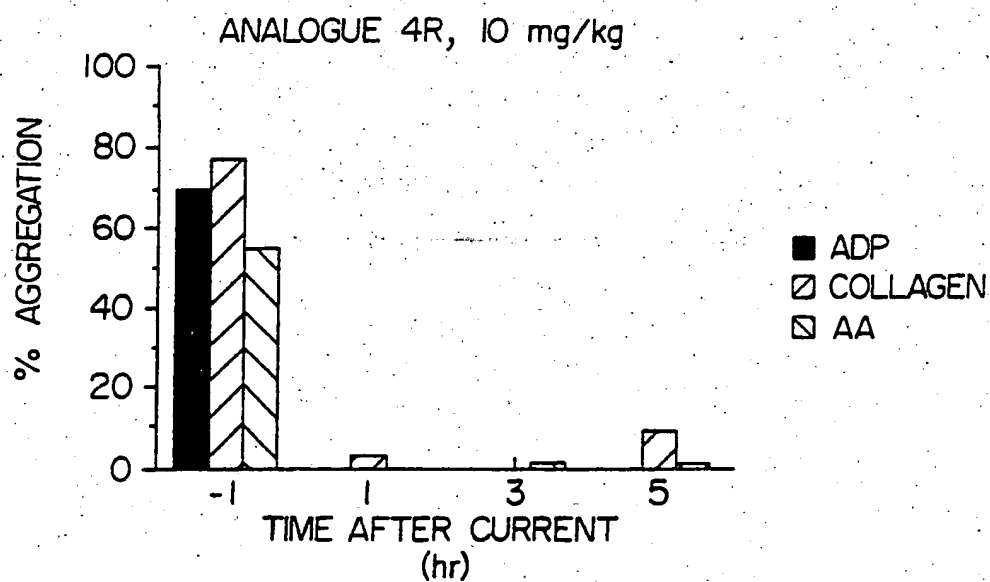


FIG. 3F

PEPTIDE PREVENTION OF CORONARY THROMBOSIS

<u>TREATMENT</u>	<u>TIME TO FULL OCCLUSION (min)^a</u>
Control	136 ± 15 (13)
Analogue 10 mg/kg	127 ± 41 (3)
Analogue 4Q 5 mg/kg	165 ± 10 (2) ^b
5 mg/kg (Low platelet count)	> 300 (2)
10 mg/kg	> 300 (1) ^c
Analogue 4R 3 mg/kg	95 (1)
10 mg/kg	> 300 (1) ^c

^a values are mean ± SEM for the number of determinations that appears in parentheses.

Statistical significance vs. control denoted as follows:

^b $p < .05$

^c $p < .01$

FIG. 4

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Effects of RGD Peptides on Hemodynamic Parameters

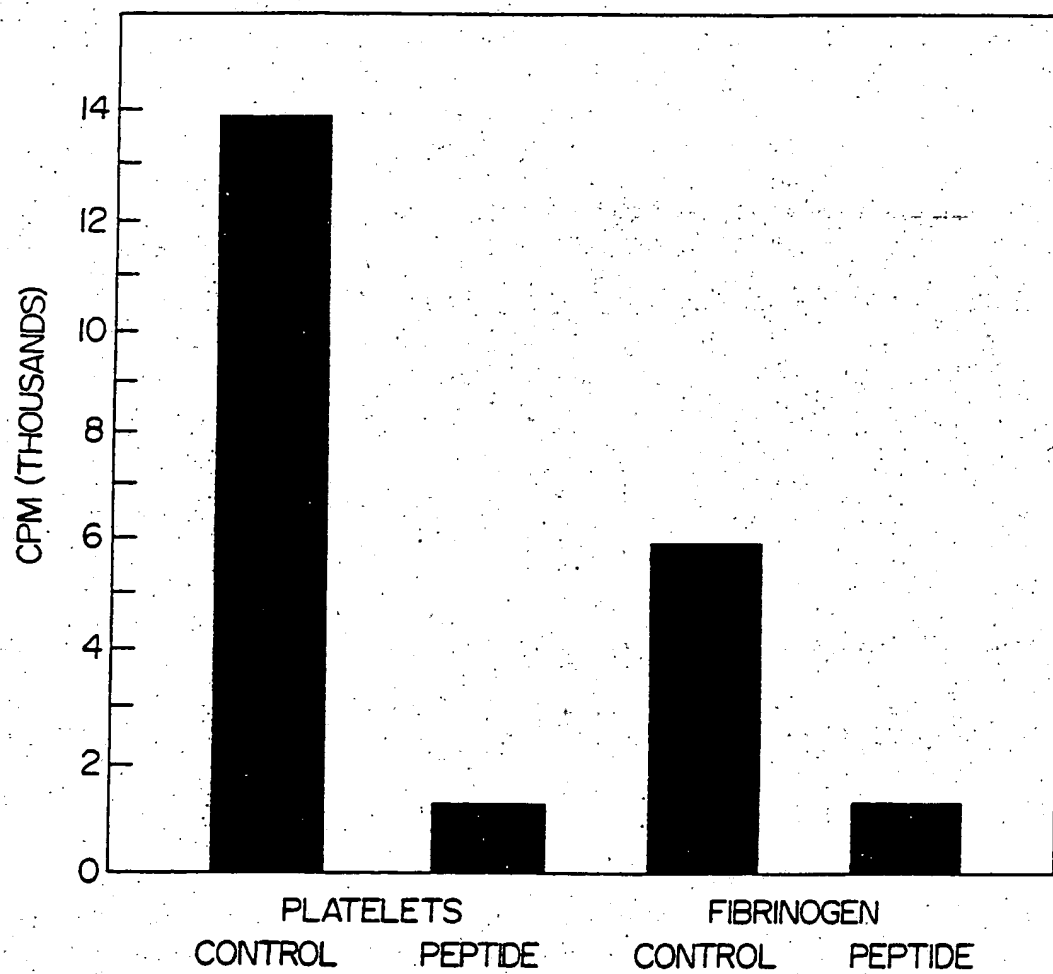
Treatment	Mean Arterial Pressure (mm Hg)	Heart Rate (Beats/Min)	Platelet Count ($10^3/\text{ml}$)	Bleeding Time (Min)
ANALOGUE 2G				
(10 mg/kg) (N=3)				
Baseline	104 \pm 5	160 \pm 10	480 \pm 12	2.9 \pm 0.6
1 hr.	96 \pm 7	172 \pm 8	409 \pm 19	2.8 \pm 0.3
3 hr.	ND	ND	395 \pm 36	2.6 \pm 0.1
5 hr.	ND	ND	556 \pm 16	2.6 \pm 0.1
ANALOGUE 4Q				
(5 mg/kg) (N=2)				
Baseline	103 \pm 3	158 \pm 9	316 \pm 52	2.8 \pm 0.3
1 hr.	107 \pm 6	160 \pm 10	406 \pm 185	2.8 \pm 0.3
3 hr.	110 \pm 10	168 \pm 7	375 \pm 25	3.6 \pm 0.1
5 hr.	ND	ND	355 \pm 47	3.0 \pm 0.5
(10 mg/kg) (N=1)				
Baseline	100	162	307	4.0
1 hr.	104	170	420	7.5
3 hr.	105	160	379	5.5
5 hr.	107	165	290	5.5
ANALOGUE 4R				
(3 mg/kg) (N=1)				
Baseline	103	170	393	4.0
1 hr.	110	165	367	4.0
3 hr.	ND	ND	420	4.0
5 hr.	ND	ND	575	5.0
(10 mg/kg) (N=1)				
Baseline	100	162	512	4.0
1 hr.	95	168	513	5.0
3 hr.	102	172	491	5.0
5 hr.	107	170	460	5.0

FIG. 5

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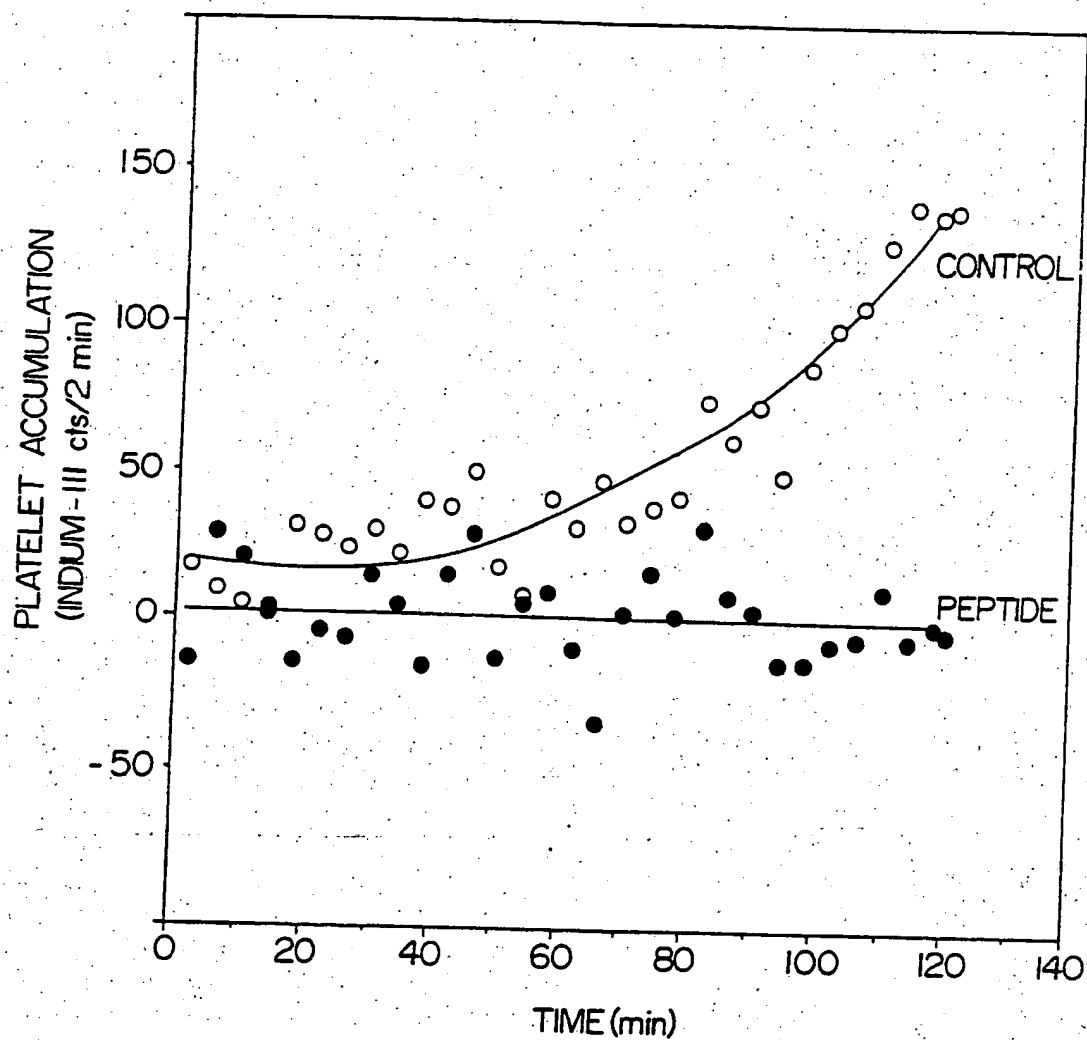
FIG. 6



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FIG. 7



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PEPTIDE EFFECTS ON ANIMAL
HEMATOLOGY & BLEEDING TIMES
(Experiment I)

<u>HEMATOLOGY PARAMETERS</u>	<u>SHUNT 1</u>		<u>SHUNT 2</u>	
	<u>0 Hr.</u>	<u>2 Hr.</u>	<u>0 Hr.</u>	<u>1 Hr.</u>
Red Blood Cells (M/UL)	4.78	4.00	4.49	4.70
White Blood Cells (K/UL)	9.3	4.0	12.0	11.4
Platelets (K/UL)	332	250	269	276
Hematocrit (%)	42	35	39	41
Hemoglobin (G/UL)	12.5	10.7	12.0	12.4
Bleeding Time* (Min)	4:48	4:55	ND	5:02
Clotting Time (Sec)	120	130	ND	ND
				124

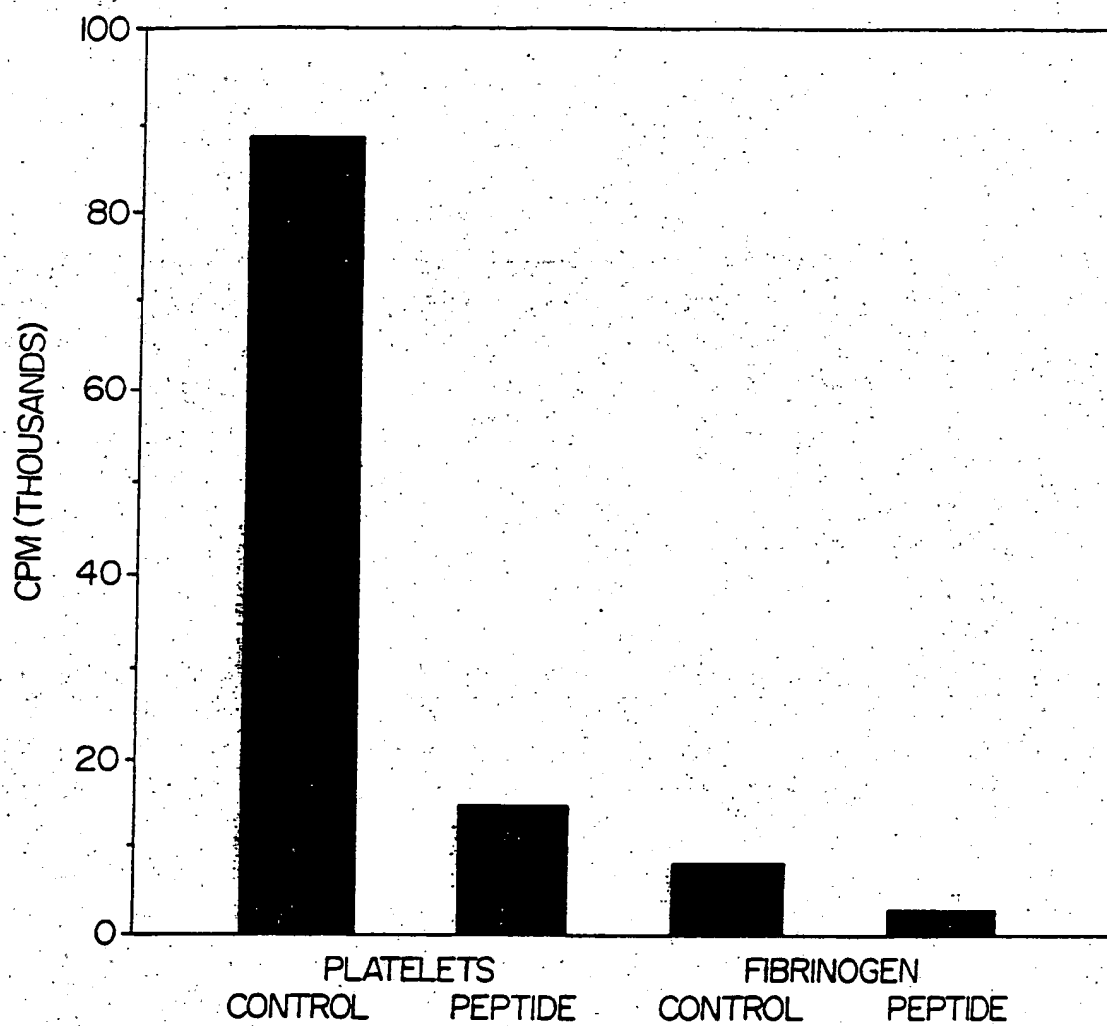
*Represents average of two simultaneous determinations.

ND = not determined.

FIG. 8

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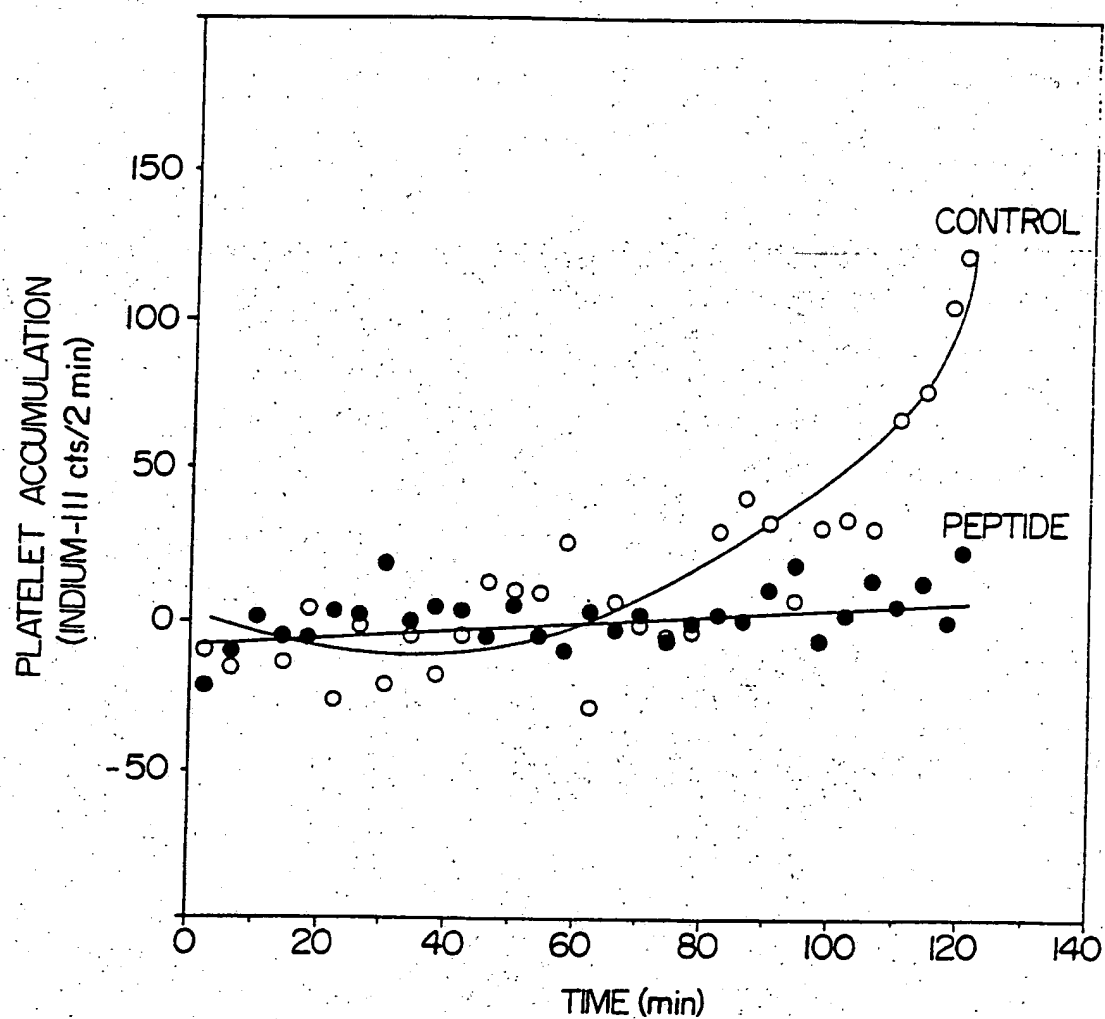
FIG. 9



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FIG. 10



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**PEPTIDE EFFECTS ON ANIMAL
HEMATOLOGY & BLEEDING TIMES
(Experiment 2)**

<u>HEMATOLOGY PARAMETERS</u>	<u>SHUNT 1</u>		<u>SHUNT 2</u>	
	<u>0 Hr.</u>	<u>2 Hr.</u>	<u>0 Hr.</u>	<u>1 Hr.</u>
Red Blood Cells (M/UL)	4.78	5.12	5.06	4.65
White Blood Cells (K/UL)	6.6	12.9	10.7	10.0
Platelets (K/UL)	274	281	281	264
Hematocrit (%)	39	41	41	37
Hemoglobin (G/UL)	12.3	13.2	12.9	12.0
Bleeding Time* (Min)	4:15	4:27	ND	4:46
Clotting Time (Sec)	131	125	ND	ND
				132

*Represents average of two simultaneous determinations.

ND = not determined.

FIG. 11

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IN VIVO AND EX VIVO EVALUATION OF PEPTIDES IN A RABBIT MODEL

CODE	Rabbit BT CBT Ratio	Rabbit Ex Vivo Plt Aggn % Inh	Rabbit PBF	Dose BOL + Inf	N	Sequence
Control	1.1	11	Normal	(Saline)	2	
8X	0.7	94	Normal	1.5+0.036	1	Ac-CNPRGD(Y-OMe)RC-NH2
8X	2.1	98	Normal	4.5+0.108	1	
11J	0.93 (dose too low)	17	Normal	1.5+0.036	1	Ac-CNPRGD(Y-OMe)EC-NH2
11K	0.69 (dose too low)	29	Normal	1.5+0.036	1	Ac-CNPRGD(Y-OMe)AC-NH2
11L	1.09 (dose too low)	12	Normal	1.5+0.036	1	Ac-CNPRGD(Y-OMe)(dR)C-NH2
11P	1.42 (dose too low)	10	Normal	1.5+0.036	1	Ac-CNPRGD(Y-OMe)OC-NH2
11Q	0.70 (dose too low)	63	Normal	1.5+0.036	1	Ac-CNPKGD(Y-OMe)RC-NH2
11R	1.40 (dose too low)	19	Normal	1.5+0.036	1	Ac-CNPRGD(Y-OMe)KC-NH2
11S	1.00 (dose too low)	10	Normal	1.5+0.036	1	Ac-CNPRGD(Y-OMe)(Clt)C-NH2
11V	1.17 (dose too low)	0	Normal	1.5 + 0.036	1	Ac-CNPRGD(Y-OMe)(Nle)C-NH2
4Q	1.4	97	Decreased	3.75+0.090	2	RPenGHRGDWRCR
4Q	1.4	99	Decreased	11.4+0.270	1	
5Q	1.3	27	ND	0.27+0.007	1	RPmcGHRGD(Y-OMc)RCR
5Q	0.75	93	Decreased	1.5+0.036	1	
7B	1.2	32	ND	0.59+0.014	1	RPmcGHRGD(p-1-F)RCR
7B	1.0	96	Decreased	3.0+0.072	2	
7T	1.1	94	Decreased	1.5+0.036	2	RPmcGHRGD(p-N02-F)RCR

Dose of [mg/kg] bolus + [mg/kg/min x 60 min] infusion

FIG. 12

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 91/02356

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶ According to International Patent Classification (IPC) or to both National Classification and IPC IPC ⁵ : C 07 K 15/00, 7/64, A 61 K 37/43																	
II. FIELDS SEARCHED <div style="text-align: center; font-size: small;">Minimum Documentation Searched ⁷</div> <table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 25%; padding: 5px;">Classification System</td> <td style="padding: 5px;">Classification Symbols</td> </tr> <tr> <td style="padding: 5px;">IPC⁵</td> <td style="padding: 5px;">C 07 K, A 61 K</td> </tr> </table> <div style="text-align: center; font-size: x-small; margin-top: 5px;">Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸</div>			Classification System	Classification Symbols	IPC ⁵	C 07 K, A 61 K											
Classification System	Classification Symbols																
IPC ⁵	C 07 K, A 61 K																
III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹ <table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 10%; padding: 5px;">Category ¹⁰</th> <th style="width: 70%; padding: 5px;">Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²</th> <th style="width: 20%; padding: 5px;">Relevant to Claim No. ¹³</th> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">X</td> <td style="padding: 5px;">WO, A, 9002751 (ASAHI GLASS CO. LTD) 22 March 1990 see claim 1 --</td> <td style="text-align: center; vertical-align: top; padding: 5px;">1</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">X</td> <td style="padding: 5px;">WO, A, 8905150 (LA JOLLA CANCER RESEARCH FOUNDATION) 15 June 1989 see claims 1-5, 11-17, 30 --</td> <td style="text-align: center; vertical-align: top; padding: 5px;">1-18</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">X</td> <td style="padding: 5px;">EP, A, 0341915 (SMITHKLINE BECKMAN CORP.) 15 November 1989 see claim 1 --</td> <td style="text-align: center; vertical-align: top; padding: 5px;">1-8</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">P, X</td> <td style="padding: 5px;">WO, A, 9015620 (COR THERAPEUTICS) 27 December 1990 see claim 32 -----</td> <td style="text-align: center; vertical-align: top; padding: 5px;">9-18</td> </tr> </table>			Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³	X	WO, A, 9002751 (ASAHI GLASS CO. LTD) 22 March 1990 see claim 1 --	1	X	WO, A, 8905150 (LA JOLLA CANCER RESEARCH FOUNDATION) 15 June 1989 see claims 1-5, 11-17, 30 --	1-18	X	EP, A, 0341915 (SMITHKLINE BECKMAN CORP.) 15 November 1989 see claim 1 --	1-8	P, X	WO, A, 9015620 (COR THERAPEUTICS) 27 December 1990 see claim 32 -----	9-18
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X	EP, A, 0341915 (SMITHKLINE BECKMAN CORP.) 15 November 1989 see claim 1 --	1-8															
P, X	WO, A, 9015620 (COR THERAPEUTICS) 27 December 1990 see claim 32 -----	9-18															
<div style="font-size: x-small;"> <div style="display: flex; justify-content: space-between;"> <div style="width: 48%;"> <p>¹⁰ Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 48%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"Δ" document member of the same patent family</p> </div> </div> </div>																	
IV. CERTIFICATION <table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 50%; padding: 5px;"> Date of the Actual Completion of the International Search 19th July 1991 </td> <td style="width: 50%; padding: 5px;"> Date of Mailing of this International Search Report 16 SEP 1991 </td> </tr> <tr> <td style="padding: 5px;"> International Searching Authority EUROPEAN PATENT OFFICE </td> <td style="padding: 5px;"> Signature of Authorized Officer Mme N. KUIPER </td> </tr> </table>			Date of the Actual Completion of the International Search 19th July 1991	Date of Mailing of this International Search Report 16 SEP 1991	International Searching Authority EUROPEAN PATENT OFFICE	Signature of Authorized Officer Mme N. KUIPER											
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International Searching Authority EUROPEAN PATENT OFFICE	Signature of Authorized Officer Mme N. KUIPER																

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☒ OBSERVATION WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE ¹

This International search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claim numbers 19-24
 Authority, namely: because they relate to subject matter not required to be searched by this
 See PCT-Rule 39.1 (iv): methods for treatment of the human
 or animal body by surgery or therapy as well as diagnostic
 methods
2. ☐ Claim numbers
 with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically: because they relate to parts of the International application that do not comply
3. ☐ Claim numbers
 the second and third sentences of PCT Rule 6.4(a). because they are dependent claims and are not drafted in accordance with

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING ²

This International Searching Authority found multiple inventions in this International application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International search report covers all searchable claims of the International application
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the International application for which fees were paid, specifically claims:
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.**

US 9102356
SA 47003

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 06/09/91. The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A- 9002751	22-03-90	JP-A- 2174797 EP-A- 0406428	06-07-90 09-01-91
WO-A- 8905150	15-06-89	AU-A- 2817389 EP-A- 0394326 JP-T- 3501610	05-07-89 31-10-90 11-04-91
EP-A- 0341915	15-11-89	AU-A- 3458889 JP-A- 2062892	09-11-89 02-03-90
WO-A- 9015620	27-12-90	AU-A- 6036990	08-01-91